Degradation of cyclic diguanosine monophosphate by a hybrid two-component protein protects *Azoarcus* sp. strain CIB from toluene toxicity

Zaira Martín-Moldes[a], Blas Blázquez[a], Claudine Baraquet[b,1], Caroline S. Harwood[b,2], María T. Zamarro[a,2], and Eduardo Díaz[a,2]

[a]Department of Environmental Biology, Centro de Investigaciones Biológicas-CSIC, 28040 Madrid, Spain; and [b]Department of Microbiology, University of Washington, Seattle, WA 98195-7242

Contributed by Caroline S. Harwood, September 29, 2016 (sent for review June 24, 2016; reviewed by Rebecca E. Parales and Kai M. Thormann)

Cyclic diguanosine monophosphate (c-di-GMP) is a second messenger that controls diverse functions in bacteria, including transitions from planktonic to biofilm lifestyles, virulence, motility, and cell cycle. Here we describe TolR, a hybrid two-component system (HTCS), from the β-proteobacterium *Azoarcus* sp. strain CIB that degrades c-di-GMP in response to aromatic hydrocarbons, including toluene. This response protects cells from toluene toxicity during anaerobic growth. Whereas wild-type cells tolerated a sudden exposure to a toxic concentration of toluene, a *tolR* mutant strain or a strain overexpressing a diguanylate cyclase gene lost viability upon toluene shock. TolR comprises an N-terminal aromatic hydrocarbon-sensing Per–Arnt–Sim (PAS) domain, followed by an autokinase domain, a response regulator domain, and a C-terminal c-di-GMP phosphodiesterase (PDE) domain. Autophosphorylation of TolR in response to toluene exposure initiated an intramolecular phosphotransfer to the response regulator domain that resulted in c-di-GMP degradation. The TolR protein was engineered as a functional sensor histidine kinase ($TolR_{SK}$) and an independent response regulator ($TolR_{RR}$). This classic two-component system (CTCS) operated less efficiently than TolR, suggesting that TolR was evolved as a HTCS to optimize signal transduction. Our results suggest that TolR enables *Azoarcus* sp. CIB to adapt to toxic aromatic hydrocarbons under anaerobic conditions by modulating cellular levels of c-di-GMP. This is an additional role for c-di-GMP in bacterial physiology.

c-di-GMP | phosphotransfer | hydrocarbon tolerance | anaerobe | biodegradation

Bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a bacterial second messenger that controls diverse cellular functions including biofilm formation, motility, virulence, and cell cycle (1, 2). Most bacteria encode several to dozens of enzymes involved in the synthesis and degradation of c-di-GMP. Diguanylate cyclases (DGCs) for c-di-GMP synthesis have a characteristic GGDEF domain, and c-di-GMP–specific phosphodiesterases (PDEs) have a characteristic EAL or HD-GYP domain (1, 3). Intracellular c-di-GMP binds to transcription factors and riboswitches to modulate gene expression, and it also serves as an allosteric effector of target proteins (1–3).

The β-proteobacterium *Azoarcus* sp. strain CIB is a rice endophyte and a free-living facultative anaerobe that grows on the aromatic hydrocarbons toluene and *m*-xylene. These compounds tend to be toxic at high concentrations because they partition into cell membranes and destroy cellular integrity. *Azoarcus* sp. CIB has distinct oxygen-requiring aerobic and oxygen-sensitive anaerobic pathways for aromatic hydrocarbon degradation (4–6). Within the *bss–bbs* gene cluster responsible for the anaerobic degradation of toluene/*m*-xylene, there is a gene, *tolR* (AzCIB_4516), that has no orthologs in any of the homologous *bss–bbs* clusters described so far in other bacteria (4, 6, 7). The predicted TolR protein is a hybrid two-component system (HTCS) that includes sensor kinase (SK) and response regulator (RR) domains, an N-terminal Per–Arnt–Sim (PAS) domain, and a C-terminal EAL domain. It does not have a DNA-binding domain and thus is not predicted to control gene expression on its own. Although HTCSs are encoded in diverse bacteria, they have so far been studied only in members of the *Bacteroidetes* phylum (8–12), where they behave as transcriptional regulators that respond to carbohydrates to control expression of polysaccharide degradation genes (13).

Here we present evidence that TolR degrades c-di-GMP in response to binding toluene and that this response protects cells from toluene toxicity by a mechanism that has yet to be elucidated. We also describe steps of intramolecular phosphoryl transfer by the HTCS TolR that lead to c-di-GMP PDE activity, thus expanding our restricted knowledge of the mechanism of action of HTCSs.

Results

***tolR* Protects Anaerobic *Azoarcus* sp. CIB Cells from Toluene Toxicity.** To explore the physiological role of TolR, we constructed a *tolR* mutant and found that it grew anaerobically on 400 μM toluene with a doubling time of 19 h, compared with a doubling time of 15 h for the wild-type parent (*SI Appendix*, Fig. S1). This suggested that TolR is not essential for anaerobic toluene mineralization

Significance

Some bacteria have a remarkable ability to grow on toxic aromatic hydrocarbons under anaerobic conditions. Although bacteria have evolved cellular mechanisms to cope with hydrocarbon toxicity, it was unclear if signal transduction systems might exist to coordinate a protective response. Here we describe a hybrid two-component system, the TolR protein, from the bacterium *Azoarcus* sp. strain CIB that on binding toluene and other aromatic hydrocarbons degrades intracellular cyclic diguanosine monophosphate (c-di-GMP). A drop in c-di-GMP protects cells from toluene toxicity. C-di-GMP, found universally in bacteria, is involved in diverse functions including biofilm formation and virulence. Our finding of hydrocarbon sensing and c-di-GMP degradation by TolR for purposes of modulating hydrocarbon toxicity describes an additional role for c-di-GMP in bacterial physiology.

Author contributions: Z.M.-M., C.B., C.S.H., M.T.Z., and E.D. designed research; Z.M.-M., B.B., C.B., and M.T.Z. performed research; Z.M.-M., C.B., C.S.H., M.T.Z., and E.D. analyzed data; and Z.M.-M., C.S.H., M.T.Z., and E.D. wrote the paper.

Reviewers: R.E.P., University of California, Davis; and K.M.T., Justus-Liebig-Universität Giessen.

The authors declare no conflict of interest.

[1]Present address: Université de Toulon, Laboratoire Materiaux Polymeres Interfaces Environnement Marin, 83957 La Garde, France.

[2]To whom correspondence may be addressed. Email: ediaz@cib.csic.es, csh5@uw.edu, or mtzamarro@cib.csic.es.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1615981113/-/DCSupplemental.

but raised the possibility that the *tolR* mutant was more sensitive to the toxic effects of toluene than the wild type. To test this idea, we exposed anaerobic cells to a range of concentrations of toluene and found that cells lost substantial viability when exposed from 4 mM up to 20 mM toluene, a concentration 10 times in excess of the maximal amount of toluene that supports anaerobic growth, over a 2-h incubation (Fig. 1*A* and *SI Appendix*, Fig. S2). In contrast, the wild-type strain did not lose viability after an anaerobic toluene shock. The toluene shock phenotype of the *tolR* mutant strain was complemented when the *tolR* gene was expressed *in trans* from plasmid pIZtolR (Fig. 1*A*). No differences in toluene tolerance were observed when the wild-type and *tolR* mutant strains were exposed to a similar toluene shock under aerobic conditions.

TolR Is a Toluene-Dependent c-di-GMP PDE. An analysis of the primary structure of the TolR protein (1,049 amino acids) revealed a modular architecture with domains found in SKs and RRs fused in a single polypeptide. There is an N-terminal superdomain consisting of a PAS sensor domain and an autokinase (AK) domain with a histidine residue (His-190) that is conserved in proteins with AK domains and is thus predicted to be the phosphorylaccepting histidine residue of TolR (Fig. 1*B*). The C-terminal superdomain of the protein has a receiver (REC) domain that harbors a conserved phosphoaccepting Asp residue and an EAL domain predicted to encode a c-di-GMP PDE (Fig. 1*B*). This domain also contains a degenerate GGDEF motif of the type that has been shown to serve as a receptor domain for c-di-GMP (1, 14). The N-terminal superdomain and the REC domain of TolR show about 50% amino acid sequence identity to members of the TodS subfamily of hybrid SKs that recognize aromatic hydrocarbons (15). To determine if TolR possesses c-di-GMP PDE activity, we transferred plasmid pIZtolR to a *Pseudomonas aeruginosa* c-di-GMP reporter strain. This strain has a deletion in the *wspF* gene, which makes the WspR DGC constitutively active and causes intracellular concentrations of c-di-GMP to be high (16). This strain also has a transcriptional fusion of *lacZ* to the promoter of the *pel* operon coding for Pel exopolysaccharide biosynthesis (*Ppel–lacZ*). The *pel* promoter responds to intracellular c-di-GMP to control *pel* gene expression in *P. aeruginosa* (17). Anaerobically grown *P. aeruginosa Ppel–lacZ* reporter cells with *tolR* expressed *in trans* from the pIZtolR plasmid showed a slight reduction of the β-galactosidase levels relative to the control strain carrying the empty pIZ1016 vector. β-Galactosidase activity was significantly more reduced when cells were exposed to 100 μM toluene (Fig. 1*C*). A similar reduction of β-galactosidase levels, although toluene-independent, was observed when *PA2133*, a gene from *P. aeruginosa* that encodes a functional c-di-GMP PDE (16), was expressed *in trans* from plasmid pIZ2133 (Fig. 1*C*). Whereas *P. aeruginosa Ppel–lacZ* reporter cells containing plasmid pIZ2133 showed a reduction of β-galactosidase activity when they were grown under aerobic conditions, no reduction of β-galactosidase activity was observed

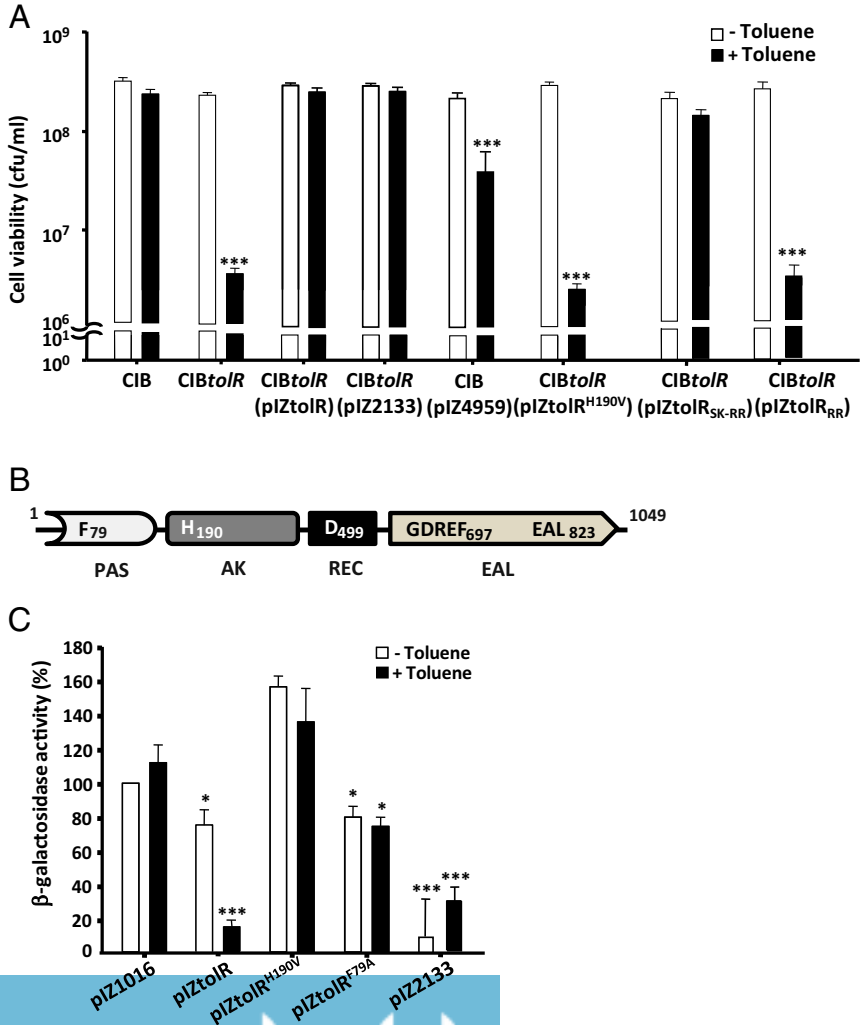

Fig. 1. TolR mediates toluene shock resistance by degrading c-di-GMP. (*A*) The effect of TolR and c-di-GMP on the resistance of *Azoarcus* sp. CIB to anaerobic toluene shock. Shown is the viability of *Azoarcus* sp. CIB wild-type (CIB) and the *tolR* mutant strain (CIB*tolR*) grown anaerobically on pyruvate in the absence (white bars) and 2 h after addition of 20 mM toluene (toluene shock) (black bars). Effects of *in trans* expression of *tolR* (pIZtolR) and various *tolR* mutants (pIZtolR^{H190V}, pIZtolR$_{SK-RR}$, pIZtolR$_{RR}$) on cell viability in the absence and after a toluene shock are also shown. Plasmid pIZ2133 expresses the *P. aeruginosa* c-di-GMP PDE PA2133; plasmid pIZ4959 expresses the *P. putida* DGC PP4959. The number of viable cells is shown as colony forming units (cfus) per milliliter. Error bars represent SD calculated from three experiments performed in duplicate. ***, significant differences between the − toluene and + toluene CIB samples with $P < 0.001$. (*B*) Diagram of the modular architecture of TolR. Key amino acids discussed in the text are indicated. The residues predicted to be phosphorylated are labeled in white. GDREF is a degenerate DGC motif. (*C*) TolR has a toluene-dependent c-di-GMP PDE activity. β-Galactosidase assays of the *P. aeruginosa PpelA::lacZ* reporter strain carrying plasmids pIZ1016 (control), pIZtolR (TolR), pIZtolR^{H190V} (TolR^{H190V}), pIZtolR^{F79A} (TolR^{F79A}), or pIZ2133 (PA2133) and cultivated anaerobically in LB medium with 1 mM IPTG in the absence (white bars) or presence (black bars) of 100 μM toluene are shown. Error bars represent SD calculated from three experiments performed in triplicate. * and ***, significant differences against the pIZ1016 control with $P < 0.1$ and $P < 0.001$, respectively.

when cells expressing the *tolR* gene *in trans* were grown aerobically (*SI Appendix*, Fig. S3). This suggests that the c-di-GMP PDE activity of TolR requires anaerobic conditions.

To test if c-di-GMP can ameliorate toluene toxicity in anaerobically grown cells of *Azoarcus* sp. CIB, we expressed *PA2133 in trans* in the *tolR* mutant and found that it restored toluene tolerance after an anaerobic toluene shock (Fig. 1*A*). On the other hand, the expression in wild-type *Azoarcus* sp. CIB of a heterologous DGC gene (*PP4959* from *Pseudomonas. putida*) (18) reduced its resistance to toluene shock (Fig. 1*A*). These results support the conclusion that intracellular levels of c-di-GMP influence the resistance of *Azoarcus* sp. CIB to toluene shock.

Toluene-Dependent Autophosphorylation of TolR Is Needed to Activate Its PDE Activity. TolR contains an AK domain (Fig. 1*B*) that may be involved in autophosphorylation. To test this, TolR protein was purified and in vitro phosphorylation assays were performed. A basal level of TolR autophosphorylation was observed, and this increased significantly (up to fivefold) when toluene was added (Fig. 2*A*, panel 1 and *SI Appendix*, Fig. S4). The substitution of the conserved His-190 residue (Fig. 1*B*) with a valine residue resulted in a TolR^{H190V} protein that when purified did not autophosphorylate either in the absence or presence of toluene (*SI Appendix*, Fig. S5), suggesting that His-190 is the site of autophosphorylation. When the TolR^{H190V} variant was expressed from plasmid pIZtolR^{H190V}, it did not have c-di-GMP PDE activity in the *P. aeruginosa* reporter strain (Fig. 1*C*) and did not confer tolerance to a toluene shock in the *Azoarcus* sp. CIB*tolR* mutant strain (Fig. 1*A*). These results indicate that autophosphorylation of TolR in response to toluene is needed to induce the c-di-GMP PDE activity of this protein.

HTCSs are characterized by an intramolecular phosphotransfer rather than by the typical intermolecular phosphotransfer of classic two-component systems (CTCSs) (19). To test this, phosphorylated TolR protein was treated with HCl, which breaks phospho-histidine bonds but not phospho-aspartate bonds (19). After HCl treatment, a phosphorylated protein was still observed (Fig. 2*A*, panel 2), but the levels of phosphorylation were reduced compared with protein treated with buffer only (Fig. 2*A*, panel 1 and *SI Appendix*, Fig. S4). Treatment with NaOH, which breaks phospho-aspartate bonds, caused only minor reduction in the levels of phosphorylated TolR protein (Fig. 2*A*, panel 3 and *SI Appendix*, Fig. S4). This pattern of phosphorylation was observed in both the presence and absence of toluene (Fig. 2*A* and *SI Appendix*, Fig. S4) and indicates that the majority of TolR contained phosphorylated AK rather than the phosphorylated REC domain. It appears that TolR undergoes intramolecular phosphotransfer from the AK to the REC domain but that there is then a rapid loss of the phosphoryl group from the latter. This is in agreement with the observed consumption of the radiolabeled ^{32}P-γ-ATP substrate and the concomitant accumulation of ^{32}Pi in the autophosphorylation reaction assay (Fig. 2*B* and *SI Appendix*, Fig. S6).

Aromatic Hydrocarbons Are Recognized by the PAS Sensor Domain of TolR. We assessed the ability of aromatic compounds in addition to toluene to stimulate autophosphorylation in vitro and found that the TolR protein recognized many different aromatic hydrocarbons including xylenes, benzene, styrene, ethylbenzene, and propylbenzene (Fig. 2*C*). Other aromatic compounds, including phenol, benzaldehyde, aniline, acetophenone, and to a minor extent 3-chlorophenol and benzyl alcohol, were also effectors. Aromatic hydrocarbons containing a branched-lateral chain [e.g., cumene (isopropylbenzene)] or compounds whose benzene ring contains a carboxy or amide group (e.g., benzoate and benzamide) or more than two substitutions [e.g., mesitylene (1,3,5-trimethylbenzene) and hydroxyquinol (benzene-1,2,4-triol)] failed to activate TolR autophosphorylation (Fig. 2*C*).

Because aromatic hydrocarbons appear to be the main effectors of the autophosphorylation activity of TolR (Fig. 2*C*), we expected that they would also activate the c-di-GMP PDE activity of this protein. To test this, the c-di-GMP PDE activity of TolR was assayed in the *P. aeruginosa Ppel–lacZ* reporter strain carrying pIZtolR and grown in the presence of different aromatic compounds. A dosage-dependent activation of β-galactosidase activity by toluene and benzene, as well as the lack of activation by benzoate, was observed (*SI Appendix*, Fig. S7).

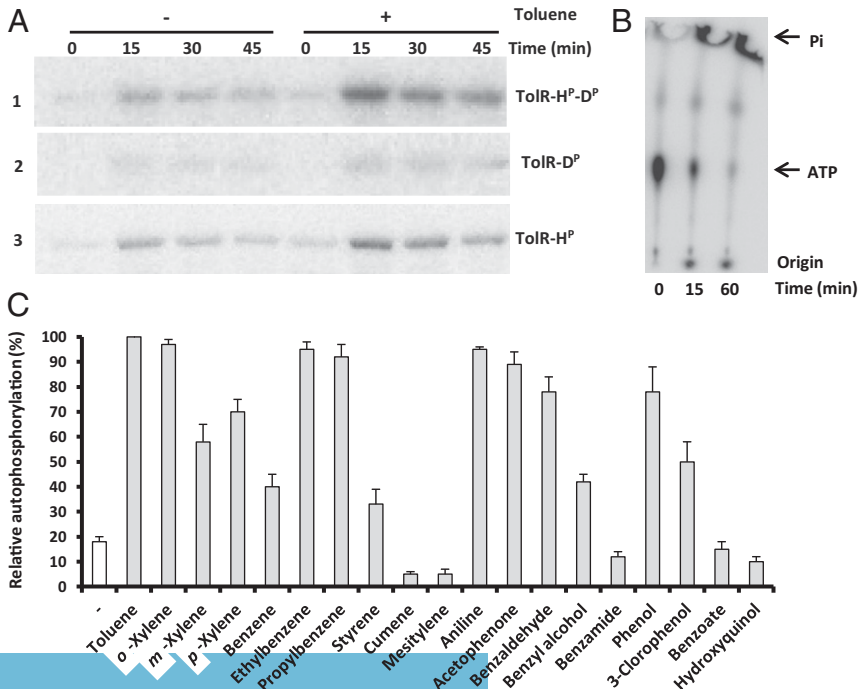

Fig. 2. Stimulation of TolR autophosphorylation by various aromatic compounds. (*A*) Acid/base sensitivity of phosphorylated TolR. Time course of autophosphorylation of TolR protein (1 μM) incubated with ^{32}P-γ-ATP in the absence (–) or presence (+) of 100 μM toluene. Identical SDS/PAGE gels containing phosphorylated proteins treated with 0.1 M Hepes buffer (pH 7.0) (TolR-H^P-D^P; panel 1), 0.1 M HCl (TolR-D^P; panel 2), or 0.2 M NaOH (TolR-H^P; panel 3). ^{32}P-labeled protein was visualized by autoradiography. (*B*) Time-course ATP consumption and Pi release by TolR protein (1 μM) incubated with ^{32}P-γ-ATP and 100 μM toluene. Samples were analyzed by TLC and subjected to phosphorimaging to examine ^{32}P-γ-ATP (ATP) consumption and ^{32}Pi (Pi) release. (*C*) TolR effector profile. Activation of the basal autophosphorylation activity was tested in vitro by incubating TolR protein (1 μM) with ^{32}P-γ-ATP in the absence (–) or presence of different aromatic compounds at 100 μM for 15 min. Phosphorylated TolR was analyzed by SDS/PAGE, and radiolabel incorporation was quantified (100% autophosphorylation is in the presence of toluene).

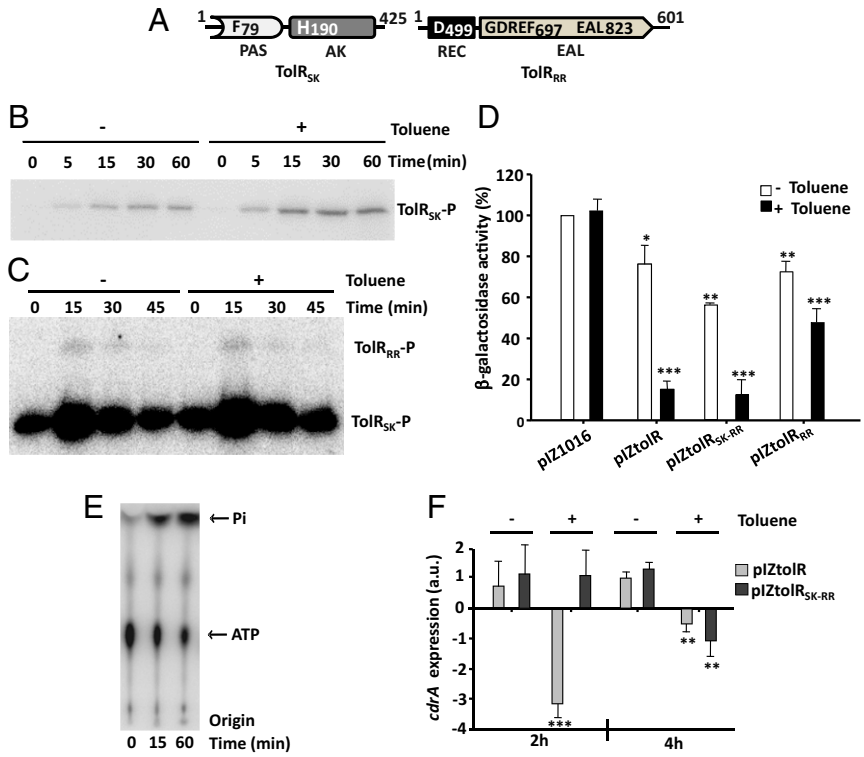

Fig. 3. Design of a functional TolR$_{SK}$/TolR$_{RR}$ CTCS and comparison with native TolR HTCS. (*A*) Scheme of the artificial TolR$_{SK}$/TolR$_{RR}$ CTCS. Domains and residues are labeled as in Fig. 1*B*. (*B*) Time course of autophosphorylation of purified TolR$_{SK}$ (0.1 μM) incubated with ^{32}P-γ-ATP in the absence (–) or presence (+) of 100 μM toluene. Samples were fractionated by SDS/PAGE, and radiolabel incorporation was detected by phosphorimaging. (*C*) TolR$_{SK}$/TolR$_{RR}$ show intermolecular transphosphorylation in vitro. Time course of transphosphorylation from TolR$_{SK}$ (2.5 μM) to TolR$_{RR}$ (2.5 μM) in the absence (–) or presence (+) of 100 μM toluene. Samples were fractionated by SDS/PAGE, and radiolabel incorporation was detected by phosphorimaging. (*D*) The TolR$_{SK}$/TolR$_{RR}$ CTCS shows toluene-dependent c-di-GMP PDE activity. Shown are β-galactosidase assays in *P. aeruginosa PpelA-lacZ* cells containing plasmids pIZ1016 (control), pIZtolR (TolR), pIZtolR$_{SK–RR}$ (TolR$_{SK}$/TolR$_{RR}$), or pIZtolR$_{RR}$ (TolR$_{RR}$) cultivated anaerobically in LB medium with 1 mM IPTG in the absence (white bars) or presence (black bars) of 100 μM toluene. Error bars represent SD calculated from three experiments performed in triplicate. *, **, and ***, significant differences against control with $P < 0.1$, $P < 0.01$, and $P < 0.001$, respectively. (*E*) Time course of ATP consumption and Pi release by purified TolR$_{SK}$ (1 μM) plus TolR$_{RR}$ (1 μM) incubated with ^{32}P-γ-ATP and 100 μM toluene. Samples were analyzed by TLC and subjected to phosphorimaging to examine ^{32}P-γ-ATP (ATP) consumption and ^{32}Pi (Pi) release. (*F*) Comparison of the c-di-GMP PDE activity of TolR and TolR$_{SK}$/TolR$_{RR}$ after a short exposure of cells to toluene. Total RNA was isolated from *P. aeruginosa* Δ*wspF* cells containing plasmid pIZtolR (gray bars) or pIZtolR$_{SK–RR}$ (black bars) grown anaerobically for 2 h or 4 h in LB medium with 1 mM IPTG in the absence (–) or presence (+) of 100 μM toluene. The relative expression of the *cdrA* gene, shown in arbitrary units, was monitored by real-time RT-PCR. Error bars represent SD calculated from three experiments performed in triplicate. ** and ***, significant differences in the presence of toluene with $P < 0.01$ and $P < 0.001$, respectively.

It is generally agreed that bacteria modulate intracellular c-di-GMP in response to environmental cues, and a few input signals sensed by enzymes involved in c-di-GMP metabolism have been identified (20–25). With the exception of oxygen, however, there are few examples of direct binding of ligands to c-d-GMP–modulating proteins. To obtain further evidence that toluene binds directly to TolR, a 3D model of its N-terminal PAS sensor domain was generated, and docking experiments with toluene were carried out (*SI Appendix*, Fig. S8). The model predicted a hydrophobic cavity at a position analogous to that of the postulated ligand-binding pocket of the PAS-1 sensor domain of TodS and similar aromatic hydrocarbon-responding SKs (e.g., TmoS, StyS, TutC) (15, 26) (*SI Appendix*, Fig. S8). Because the aromatic side chain of Phe-79 in TodS was shown to play a central role in the recognition of a broad series of ligands (15), we checked whether the equivalent Phe-79 residue in TolR (*SI Appendix*, Fig. S8) could have a similar role by replacing it with an alanine residue to generate a TolR^{F79A} variant. The c-di-GMP PDE activity of TolR^{F79A} did not increase in response to toluene (Fig. 1*C*), suggesting that Phe-79 is a critical residue for the interaction of the PAS sensor domain of TolR with its effector molecules. Thus, the PAS sensor domain of TolR likely contains an aromatic compound ligand-binding pocket, as has been previously shown for the PAS-1 domain of the aromatic hydrocarbon-responding hybrid SK TodS (15).

Evolving TolR Toward a Functional CTCS. The TolR protein may constitute an example of a recent evolutionary event where a hydrocarbon-responding SK became fused to a RR protein with c-di-GMP PDE activity. We constructed a putative CTCS precursor of TolR by engineering a SK component, TolR$_{SK}$, and a separate RR component, TolR$_{RR}$ (Fig. 3*A*). The TolR$_{SK}$ protein autophosphorylated, and a twofold stimulation of autophosphorylation by toluene was seen (Fig. 3*B* and *SI Appendix*, Fig. S9). However, whereas the TolR protein was maximally autophosphorylated after 15 min and then showed a decrease of the radioactive signal (Fig. 2*A* and *SI Appendix*, Fig. S4), the TolR$_{SK}$ protein did not show a decay of the radiactive signal even after 60 min of incubation (Fig. 3*B* and *SI Appendix*, Fig. S9). When TolR$_{SK}$ was incubated with purified TolR$_{RR}$, transphosphorylation to TolR$_{RR}$ was observed, and some decay of the radioactive signal from TolR$_{SK}$ was detected in both the presence and absence of toluene (Fig. 3*C* and *SI Appendix*, Fig. S10). Thus, TolR$_{SK}$/TolR$_{RR}$ constitute a functional CTCS where toluene appears to stimulate the autophosphorylation of TolR$_{SK}$ rather than reducing the dephosphorylation rate of TolR$_{RR}$.

To check whether the artificial $TolR_{SK}/TolR_{RR}$ CTCS was also functional in vivo in modulating the cellular level of c-di-GMP, we constructed plasmids $pIZtolR_{SK–RR}$, which expresses the $tolR_{SK}$ and $tolR_{RR}$ genes, and $pIZtolR_{RR}$, which expresses only the $tolR_{RR}$ gene. Anaerobically grown *P. aeruginosa Ppel–lacZ* reporter cells containing the $pIZtolR_{SK–RR}$ plasmid showed a reduction of β-galactosidase levels with respect to those of the control strain containing the pIZ1016 vector, and this reduction was significantly increased in the presence of toluene, as was observed in *P. aeruginosa* cells expressing the *tolR* gene *in trans* (Fig. 3*D*). *P. aeruginosa Ppel–lacZ* reporter cells containing the $pIZtolR_{RR}$ plasmid did not show a reduction of the β-galactosidase activity in the presence of toluene as great as that observed with the complete $TolR_{SK}/TolR_{RR}$ CTCS (Fig. 3*D*), suggesting that an efficient c-di-GMP PDE activity requires phosphorylation of the aspartic acid at the REC domain by the $TolR_{SK}$ SK. As expected from this, the $TolR_{SK}/TolR_{RR}$ system, but not $TolR_{RR}$ alone, was able to restore toluene tolerance to the *Azoarcus* sp. CIB*tolR* mutant (Fig. 1*A*).

TolR Is More Efficient Than $TolR_{SK}/TolR_{RR}$. To compare the efficiency of intramolecular versus intermolecular phosphoryl transfer, we performed an in vitro phosphorylation reaction assay of the $TolR_{SK}/TolR_{RR}$ CTCS, and we compared the time course of radiolabeled ^{32}P-γ-ATP consumption and accumulation of released ^{32}Pi with that previously determined for the native TolR protein. Whereas the TolR protein consumed most of the radiolabeled ^{32}P-γ-ATP substrate after 60 min (Fig. 2*B*), 40% of the initial amount of ATP substrate remained after 60 min in the $TolR_{SK}/TolR_{RR}$ reaction assay (Fig. 3*E* and *SI Appendix*, Fig. S11). This is consistent with the notion that intramolecular phosphotransfer in TolR is more efficient than the intermolecular phosphotransfer between $TolR_{SK}$ and $TolR_{RR}$.

To further explore whether the apparently more efficient intramolecular phosphotransfer in TolR leads to more rapid c-di-GMP degradation relative to that derived from the $TolR_{SK}/TolR_{RR}$ CTCS, we compared the c-di-GMP PDE activity of both systems after exposure to toluene. This involved monitoring the expression of *cdrA*, an adhesion gene that is positively regulated by c-di-GMP in *P. aeruginosa* (27, 28). *P. aeruginosa* Δ*wspF* cells (which have relatively high intracellular c-di-GMP levels) containing either plasmid pIZtolR or plasmid $pIZtolR_{SK–RR}$ were grown anaerobically in the presence or absence of toluene, and their relative c-di-GMP levels were estimated by measuring the expression of *cdrA* by RT-PCR. Whereas the expression of *cdrA* was similar in both strains in the absence of toluene, the cells expressing the *tolR* gene showed a clear reduction of *cdrA* transcript levels after 2 h of exposure to toluene. A modest reduction of *cdrA* transcript levels was observed in the cells expressing the $TolR_{SK}/TolR_{RR}$ CTCS but only after 4 h of toluene exposure (Fig. 3*F*). This suggests that the c-di-GMP PDE activity of TolR is more active than that of the $TolR_{SK}/TolR_{RR}$ CTCS.

Discussion

Here we show that the HCTS TolR from the β-proteobacterium *Azoarcus* sp. CIB functions to protect cells from the deleterious effects of toluene by degrading intracellular c-di-GMP (Fig. 1). Our data are consistent with a model (Fig. 4) where a conformational change triggered by binding of toluene to TolR causes autophosphorylation and intramolecular phosphotransfer reactions that result in a stimulation of the c-di-GMP PDE activity of its C-terminal EAL domain under anaerobic conditions. Decreased intracellular c-di-GMP levels resulting either from TolR stimulation or by overexpression of a cloned PDE gene protect cells from toluene toxicity (Fig. 1). The most direct toxic effects of toluene occur at the bacterial cytoplasmic membrane by disorganizing its structure, releasing lipids and proteins, and hence impairing vital functions (29, 30). Bacterial responses to toluene toxicity include fine-tuning of lipid fluidity at the membrane,

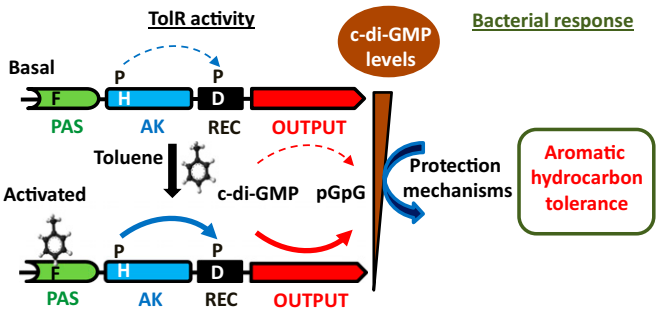

Fig. 4. Model for mechanism of action of the TolR HTCS in mediating a drop in intracellular c-di-GMP leading to toluene toxicity tolerance in *Azoarcus* sp. CIB. In the absence of aromatic hydrocarbons, autophosphorylation and intramolecular phosphotransfer support a basal level of TolR c-di-GMP PDE activity. In the presence of aromatic hydrocarbons (e.g., toluene) that bind to the PAS domain of TolR, autophosphorylation and intramolecular phosphotransfer significantly increase and lead to a stimulation of c-di-GMP PDE activity. A decrease in levels of intracellular c-di-GMP triggers a protective response to the deleterious effects of high concentrations of aromatic hydrocarbons when *Azoarcus* sp. CIB is cultivated under anaerobic conditions.

activation of the general stress response, increased energy generation, and induction of specific efflux pumps to extrude the hydrocarbon to the medium (30, 31). We do not know how intracellular depletion of c-di-GMP results in protection of *Azoarcus* strain CIB from toluene toxicity. C-di-GMP can act at the transcriptional, posttranscriptional, and/or posttranslational levels to modulate cell physiology (1–3). Examples of posttranslational effects of c-di-GMP include decreases in flagellar rotation and increases in polysaccharide synthesis in response to elevated c-di-GMP (1–3). C-di-GMP can bind to a variety of different types of proteins to modulate their activities. Among these are proteins with PilZ domains (pfam07238) (1–3). *Azoarcus* strain CIB has five genes annotated as encoding PilZ domain-containing proteins. It is worth noting that one of these (AzCIB_3987) lies next to genes predicted to encode an efflux pump.

TolR responds to toluene concentrations as low as 100 μM (Fig. 1*C*). It is likely that it can confer protection to toluene toxicity in this concentration range because an *Azoarcus* sp. CIB*tolR* mutant does not grow as fast with 400 μM toluene as a sole carbon and energy source as its wild-type parent (*SI Appendix*, Fig. S1). We found that high concentrations of 10–20 mM toluene were necessary to demonstrate the toluene shock response that caused the *tolR* mutant to lose viability. Cells present at toxic waste sites or near natural hydrocarbon seeps may encounter pockets of undissolved aromatic hydrocabons, and the toluene protective response mediated by TolR would be a major advantage in these situations. TolR mediates its protective response to toluene only when *Azoarcus* sp. CIB is grown anaerobically, which agrees with the fact that the *tolR* gene is located within the *bss–bbs* cluster responsible for anaerobic degradation of toluene (6). Moreover, when expressed heterologously in *P. aeruginosa*, TolR responds to toluene to degrade c-di-GMP only under anaerobic conditions (*SI Appendix*, Fig. S3). These results suggest that TolR is sensitive to oxygen. The mechanistic basis for this will be the subject of future work.

HTCSs are encoded in the genomes of phylogenetically diverse bacteria including members of the *Chloroflexi* phylum and some γ- and δ-proteobacteria, and they are especially prevalent in *Bacteroides* (9, 32). Membrane-spanning HTCSs from *Bacteroides thetaiotaomicron* detect carbohydrates through an extracellular sensor domain, leading to the activation of an output domain that functions as a transcriptional regulator to control expression of polysaccharide utilization gene clusters (13). TolR by contrast is a soluble protein that controls c-di-GMP production rather than gene expression in response to binding a range of

different aromatic compounds at a single ligand-binding site located in its N-terminal PAS domain. Activation of an enzymatic activity is expected to occur more rapidly than a transcriptional response, and in the case of *Azoarcus* sp. CIB, this may be important for countering the immediate damaging effects that toluene and other aromatic hydrocarbons have on membrane integrity.

We found that TolR retained its function as a toluene-responsive PDE when we converted it to a CTCS ($TolR_{SK}/TolR_{RR}$), although it did not operate as efficiently when the two domains were separated (Fig. 3*D*). $TolR_{SK}/TolR_{RR}$ showed a less efficient phosphotransfer (Fig. 3 *B* and *E*) and a slower rate of c-di-GMP PDE activity (Fig. 3*F*) than the TolR protein. This is consistent with previous suggestions from work with *Bacteroides* that in comparison with CTCSs, HTCSs may provide a more specific and efficient intramolecular phosphoryl transfer reaction as well as enable rapid dephosphorylation upon signaling cessation (19, 33, 34).

Materials and Methods

Bacterial Strains, Plasmids, Growth Conditions, and Molecular Biology. The bacterial strains and plasmids used in this study are listed in *SI Appendix*, Table S1. The construction of recombinant plasmids, $TolR^{F79A}$ and $TolR^{H190V}$ mutants, and the *Azoarcus* sp. CIB*tolR* mutant strain are detailed in *SI Appendix, SI Materials and Methods*. Standard molecular biology techniques were performed as previously described (35, 36). *Escherichia coli* and *P. aeruginosa* PAO1 cells were routinely grown, either aerobically or anaerobically (using 10 mM nitrate as the terminal electron acceptor), at 37 °C in Lysogeny Broth (LB) medium (35). *Azoarcus* strains were grown anaerobically at 30 °C in Medio Completo (MC) medium containing the indicated carbon source as described previously (37). Plasmids were transferred to *E. coli* and *P. aeruginosa* strains by electroporation (GenePulser; BioRad) and to *Azoarcus* strains by biparental filter mating (37).

Enzyme Assays. The β-galactosidase activities of the *P. aeruginosa Ppel–lacZ* reporter strain were measured with midexponential phase cultures of permeabilized cells, as previously described (17). The autophosphorylation and phosphoryl transfer assays were performed as detailed in *SI Appendix, SI Materials and Methods*. Protein samples were analyzed by SDS/PAGE gels, and radiolabel incorporation was detected by autoradiography. ^{32}P-γ-ATP consumption and ^{32}Pi release were analyzed by polyethyleneimine–cellulose TLC and autoradiography.

Toluene Shock Assay. *Azoarcus* strains were grown anaerobically in MC medium containing 0.2% pyruvate and, when required, 1 mM IPTG. When cultures reached midexponential phase, they were divided into two, and 20 mM toluene was added to one half and the other half was used as the control. Cultures were then incubated with shaking for 2 additional hours, and the number of viable cells was determined by plating on 0.2% succinate-containing MC medium plates.

Other Techniques. The overproduction and purification of TolR and its derivatives were carried out as indicated in *SI Appendix, SI Materials and Methods*. Proteins were analyzed by SDS/PAGE and Coomassie stained as described previously (35). RNA extraction and real-time RT-PCR studies are detailed in *SI Appendix, SI Materials and Methods*. The 3D modeling of the PAS domain of TolR and docking experiments were performed as indicated in *SI Appendix, SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank A. Valencia for technical assistance, L. Agulló for the construction of plasmid pIZ4959, Secugen S.L. for DNA sequencing, R. Matesanz for the modeling and docking analyses, and J.L. García for inspiring discussions and the critical reading of the manuscript. This work was supported by Ministry of Economy and Competitiveness of Spain Grant BIO2012-39501, European Union FP7 Grant 311815, and Fundación Ramón-Areces XVII CN (to E.D.) and NIH Grant R01-GM56665 (to C.S.H.). Z.M.-M. was the recipient of a Research Personnel Formation (FPI) fellowship from the Ministry of Economy and Competitiveness of Spain.

1. Römling U, Galperin M-Y, Gomelsky M (2013) Cyclic di-GMP: The first 25 years of a universal bacterial second messenger. *Microbiol Mol Biol Rev* 77(1):1–52.
2. Valentini M, Filloux A (2016) Biofilms and cyclic di-GMP (c-di-GMP) signaling: Lessons from *Pseudomonas aeruginosa* and other bacteria. *J Biol Chem* 291(24):12547–12555.
3. Krasteva PV, Giglio KM, Sondermann H (2012) Sensing the messenger: The diverse ways that bacteria signal through c-di-GMP. *Protein Sci* 21(7):929–948.
4. Carmona M, et al. (2009) Anaerobic catabolism of aromatic compounds: A genetic and genomic view. *Microbiol Mol Biol Rev* 73(1):71–133.
5. Fernández H, et al. (2014) *Azoarcus* sp. CIB, an anaerobic biodegrader of aromatic compounds shows an endophytic lifestyle. *PLoS One* 9(10):e110771.
6. Martín-Moldes Z, et al. (2015) Whole-genome analysis of *Azoarcus* sp. strain CIB provides genetic insights to its different lifestyles and predicts novel metabolic features. *Syst Appl Microbiol* 38(7):462–471.
7. Kim SJ, et al. (2014) An uncultivated nitrate-reducing member of the genus *Herminiimonas* degrades toluene. *Appl Environ Microbiol* 80(10):3233–3243.
8. Hasegawa Y, et al. (2003) A novel type of two-component regulatory system affecting gingipains in *Porphyromonas gingivalis*. *Microbiol Immunol* 47(11):849–858.
9. Sonnenburg ED, et al. (2006) A hybrid two-component system protein of a prominent human gut symbiont couples glycan sensing in vivo to carbohydrate metabolism. *Proc Natl Acad Sci USA* 103(23):8834–8839.
10. Sonnenburg ED, et al. (2010) Specificity of polysaccharide use in intestinal bacteroides species determines diet-induced microbiota alterations. *Cell* 141(7):1241–1252.
11. Niwa D, Nishikawa K, Nakamura H (2011) A hybrid two-component system of *Tannerella forsythia* affects autoaggregation and post-translational modification of surface proteins. *FEMS Microbiol Lett* 318(2):189–196.
12. Hirano T, et al. (2013) Regulon controlled by the GppX hybrid two component system in *Porphyromonas gingivalis*. *Mol Oral Microbiol* 28(1):70–81.
13. Lowe EC, Baslé A, Czjzek M, Firbank SJ, Bolam D-N (2012) A scissor blade-like closing mechanism implicated in transmembrane signaling in a *Bacteroides* hybrid two-component system. *Proc Natl Acad Sci USA* 109(19):7298–7303.
14. Christen M, Christen B, Folcher M, Schauerte A, Jenal U (2005) Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. *J Biol Chem* 280(35):30829–30837.
15. Busch A, Lacal J, Martos A, Ramos JL, Krell T (2007) Bacterial sensor kinase TodS interacts with agonistic and antagonistic signals. *Proc Natl Acad Sci USA* 104(34):13774–13779.
16. Hickman JW, Tifrea DF, Harwood CS (2005) A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc Natl Acad Sci USA* 102(40):14422–14427.
17. Baraquet C, Murakami K, Parsek MR, Harwood CS (2012) The FleQ protein from *Pseudomonas aeruginosa* functions as both a repressor and an activator to control gene expression from the *pel* operon promoter in response to c-di-GMP. *Nucleic Acids Res* 40(15):7207–7218.
18. Matilla MA, Travieso ML, Ramos JL, Ramos-González MI (2011) Cyclic diguanylate turnover mediated by the sole GGDEF/EAL response regulator in Pseudomonas putida: Its role in the rhizosphere and an analysis of its target processes. *Environ Microbiol* 13(7):1745–1766.
19. Townsend GE, 2nd, Raghavan V, Zwir I, Groisman EA (2013) Intramolecular arrangement of sensor and regulator overcomes relaxed specificity in hybrid two-component systems. *Proc Natl Acad Sci USA* 110(2):E161–E169.
20. Chen AI, et al. (2014) *Candida albicans* ethanol stimulates *Pseudomonas aeruginosa* WspR-controlled biofilm formation as part of a cyclic relationship involving phenazines. *PLoS Pathog* 10(10):e1004480.
21. Irie Y, et al. (2012) Self-produced exopolysaccharide is a signal that stimulates biofilm formation in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* 109(50):20632–20636.
22. Liu N, et al. (2012) Nitric oxide regulation of cyclic di-GMP synthesis and hydrolysis in *Shewanella woodyi*. *Biochemistry* 51(10):2087–2099.
23. Chua SL, et al. (2015) C-di-GMP regulates *Pseudomonas aeruginosa* stress response to tellurite during both planktonic and biofilm modes of growth. *Sci Rep* 5:10052.
24. Tuckerman JR, et al. (2009) An oxygen-sensing diguanylate cyclase and phosphodiesterase couple for c-di-GMP control. *Biochemistry* 48(41):9764–9774.
25. Wan X, et al. (2009) Globins synthesize the second messenger bis-(3′-5′)-cyclic diguanosine monophosphate in bacteria. *J Mol Biol* 388(2):262–270.
26. Krell T, et al. (2010) Bacterial sensor kinases: Diversity in the recognition of environmental signals. *Annu Rev Microbiol* 64:539–559.
27. Borlee BR, et al. (2010) *Pseudomonas aeruginosa* uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. *Mol Microbiol* 75(4):827–842.
28. Baraquet C, Harwood CS (2015) FleQ DNA binding consensus sequence revealed by studies of FleQ-dependent regulation of biofilm gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* 198(1):178–186.
29. Domínguez-Cuevas P, González-Pastor JE, Marqués S, Ramos JL, de Lorenzo V (2006) Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas putida* KT2440 cells exposed to toluene. *J Biol Chem* 281(17):11981–11991.
30. Ramos JL, et al. (2015) Mechanisms of solvent resistance mediated by interplay of cellular factors in *Pseudomonas putida*. *FEMS Microbiol Rev* 39(4):555–566.
31. Trautwein K, et al. (2008) Solvent stress response of the denitrifying bacterium "*Aromatoleum aromaticum*" strain EbN1. *Appl Environ Microbiol* 74(8):2267–2274.
32. Schneiker S, et al. (2007) Complete genome sequence of the myxobacterium *Sorangium cellulosum*. *Nat Biotechnol* 25(11):1281–1289.
33. Raghavan V, Groisman EA (2010) Orphan and hybrid two-component system proteins in health and disease. *Curr Opin Microbiol* 13(2):226–231.
34. Lynch JB, Sonnenburg JL (2012) Prioritization of a plant polysaccharide over a mucus carbohydrate is enforced by a Bacteroides hybrid two-component system. *Mol Microbiol* 85(3):478–491.
35. Sambrook J, Rusell DW (2001) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
36. Moreno-Ruiz E, Hernáez MJ, Martínez-Pérez O, Santero E (2003) Identification and functional characterization of *Sphingomonas macrogolitabida* strain TFA genes involved in the first two steps of the tetralin catabolic pathway. *J Bacteriol* 185(6):2026–2030.
37. López Barragán MJ, et al. (2004) The *bzd* gene cluster, coding for anaerobic benzoate catabolism, in *Azoarcus* sp. strain CIB. *J Bacteriol* 186(17):5762–5774.