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To cite this version:
Suguru Yamasaki, Ryosuke Nakashima, Keisuke Sakurai, Sylvie Baucheron, Etienne Giraud, et al.. Crystal structure of the multidrug resistance regulator RamR complexed with bile acids. Scientific Reports, Nature Publishing Group, 2019, 9 (1), pp.177. 10.1038/s41598-018-36025-8. hal-02619060

HAL Id: hal-02619060
https://hal.inrae.fr/hal-02619060
Submitted on 25 May 2020

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Crystal structure of the multidrug resistance regulator RamR complexed with bile acids

Suguru Yamasaki1, Ryosuke Nakashima2, Keisuke Sakurai2, Sylvie Baucheron3,4, Etienne Giraud3,4, Benoît Doublet3,4, Axel Cloeckaert3,4 & Kunihiko Nishino1,2

During infection, Salmonella senses and responds to harsh environments within the host. Persistence in a bile-rich environment is important for Salmonella to infect the small intestine or gallbladder and the multidrug efflux system AcrAB-TolC is required for bile resistance. The genes encoding this system are mainly regulated by the ramRA locus, which is composed of the divergently transcribed ramA and ramR genes. The acrAB and tolC genes are transcriptionally activated by RamA, whose encoding gene is itself transcriptionally repressed by RamR. RamR recognizes multiple drugs; however, the identity of the environmental signals to which it responds is unclear. Here, we describe the crystal structures of RamR in complexes with bile components, including cholic acid and chenodeoxycholic acid, determined at resolutions of 2.0 and 1.8 Å, respectively. Both cholic and chenodeoxycholic acids form four hydrogen bonds with Tyr59, Thr85, Ser137 and Asp152 of RamR, instead of π–π interactions with Phe155, a residue that is important for the recognition of multiple compounds including berberine, crystal violet, dequalinium, ethidium bromide and rhodamine 6 G. Binding of these compounds to RamR reduces its DNA-binding affinity, resulting in the increased transcription of ramA and acrAB-tolC. Our results reveal that Salmonella senses bile acid components through RamR and then upregulates the expression of RamR, which can lead to induction of acrAB-tolC expression with resulting tolerance to bile-rich environments.

Salmonella enterica serovar Typhimurium (S. Typhimurium) contains at least nine multidrug efflux systems. Among these, the AcrAB-TolC system, whose AcrB transporter belongs to the resistance–nodulation–cell division family, is particularly effective in generating bile acid resistance. Bile induces the expression of acrAB, and this induction is mediated by the transcriptional regulators RamA and RamR. The global transcriptional activator RamA, which belongs to the AraC/XylS family of regulatory proteins, activates the expression of acrAB and tolC genes. The local transcriptional repressor RamR, which is located directly upstream of ramA, belongs to the TetR family of regulatory proteins and represses the expression of both ramA and ramR. Additionally, it has recently been found that bile inhibits the binding of RamR to the ramA promoter region and activates ramA gene expression, resulting in the increased expression of acrAB and tolC.

Our previous biochemical and structural studies found that RamR can recognize multiple compounds, including berberine, crystal violet, dequalinium, ethidium bromide and rhodamine 6 G. Binding of these compounds to RamR results in the increased expression of ramA; all the compounds recognized by RamR are also known...
substrates of AcrAB. However, these five drug compounds are not usually present in environments inhabited by *Salmonella*, such as the intestine, and so, because bile induces *ramA* expression, we hypothesized that RamR also recognizes some components of bile acids.

There are several derivatives of bile acids. Two primary compounds, cholic (3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid; Fig. 1b) and chenodeoxycholic acids (3α, 7α-dihydroxy-5β-cholan-24-oic acid; Fig. 1b) belong to the C24 group, whose members have steroidal backbones and are metabolized from cholesterol in hepatocytes. Primary bile acids are metabolized in the liver, via conjugation to glycine or taurine. In the intestine, a broad range of intestinal anaerobic bacteria modify primary bile acids through hydrolysis and hydroxyl group dehydrogenation to produce secondary bile acids, such as deoxycholic acid (3α, 12β-dihydroxy-5β-cholan-24-oic acid; Fig. 1b) and lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid). Here, we report the crystal structure of RamR in complex with cholic and chenodeoxycholic acids. Both cholic and chenodeoxycholic acids decreased the DNA-binding affinity of RamR, resulting in increased transcription of *ramA*.

Results

Cholic and chenodeoxycholic acids induce *ramA* expression in a RamR-dependent manner. It has previously been shown that *ramA* expression is activated by a crude ox-bile extract, which is mainly dependent on RamR and is required for the bile-mediated transcriptional activation of *acrAB* and tolC genes. However, the components of bile that influence the expression level of *ramA* have, to date, remained unknown. To identify these components and investigate their possible action on RamR, we tested the effect of a crude ox-bile extract, primary bile acids (cholic and chenodeoxycholic acids), and a secondary bile acid (deoxycholic acid) on the expression level of *ramA* in the wild-type *S. Typhimurium* strain and its *ramR* deletion mutant (Fig. 1c). We confirmed that the bile extract increased the *ramA* transcript level by approximately 20-fold in the wild-type strain, but only by 3.9-fold in the *ramR* deletion mutant (Fig. 1c). Among the bile acid components tested, chenodeoxycholic and cholic acids increased *ramA* expression by more than 5.0-fold in the wild-type strain (7.2- and 5.6-fold, respectively; Fig. 1c), but not in the mutant (a 0.8-fold reduction and a 1.6-fold increase, respectively; Fig. 1c). These results indicate that *ramA* induction by cholic and chenodeoxycholic acids is dependent on RamR. In contrast, deoxycholic acid increased *ramA* expression only slightly; this effect appeared to be independent of RamR, as it was similar in the wild-type strain (1.7-fold increase) and in the *ramR* deletion mutant (2.7-fold increase).

Binding of bile acids to RamR. Because cholic and chenodeoxycholic acids induce *ramA* in a RamR-dependent manner, we hypothesized that these compounds are recognized by RamR. To test the possibility that cholic and chenodeoxycholic acids may bind to RamR, we employed surface plasmon resonance (SPR) analysis using a Biacore T200 instrument (GE Healthcare). Cholic and chenodeoxycholic acids were separately passed over RamR immobilized on a CM5 sensor chip and the SPR responses indicated that both bound directly...
Figure 2. Co-crystal structures of RamR with cholic and chenodeoxycholic acids. (a) Full structure of the RamR dimer bound to two molecules of cholic or chenodeoxycholic acid. The α-helices in RamR are indicated as α1, α2, α3, α4, α5, α6, α7a, α7b, α8a, α8b and α9. α7b and α8a are uncoiled upon the binding of cholic or chenodeoxycholic acid. 2Fo−Fc electron densities for cholic and chenodeoxycholic acids are show as a blue mesh contoured at 1.2σ. The carbon atoms of cholic or chenodeoxycholic acid are shown in cyan and oxygen atoms are shown in red. (b) Close-up view of the ligand binding site. Electron density map of the protein moiety (red mesh) and cholic and chenodeoxycholic acids (blue mesh) are contoured at 1.2σ. Carbon atoms of bile acid components and RamR are shown in cyan and green, respectively. Nitrogen, oxygen and sulfur atoms are shown in blue, red and yellow, respectively. The presence of a 12-hydroxyl group in cholic acid and its absence in chenodeoxycholic acid are indicated by red arrows. (c) Comparison of the unligated RamR structure (indicated as a red ribbon, PDB ID: 3VVX) with the ligated (cholic acid: 6IE8 or chenodeoxycholic acid: 6IE9) structures (indicated as green ribbons). 2Fo−Fc electron density for cholic acid and chenodeoxycholic acid is shown as a
to RamR, with the responses increasing in a concentration-dependent manner (Fig. 1d). The $K_D$ values obtained from SPR data for chenodeoxycholic and cholic acids were 65.3 µM and 60.5 µM, respectively.

**Co-crystal structures of RamR with cholic acid and chenodeoxycholic acid.** To elucidate the recognition mechanism of cholic and chenodeoxycholic acids by RamR, we determined the individual crystal structures of RamR complexed to both of these compounds (Fig. 2a). Electron density maps of each co-crystal structure are shown in Fig. 2b and these structures were refined to resolutions of 2.0 and 1.8 Å for cholic and chenodeoxycholic acids, respectively (Supplementary Table S1). As shown in Fig. 2a, RamR binds two molecules of either cholic or chenodeoxycholic acid per dimer. Both compounds were completely enclosed by the RamR-binding pockets. The structural difference between cholic and chenodeoxycholic acid lies in the presence or absence of a 12-hydroxyl group (Fig. 1b), which in turn results in a difference in the electron density maps of RamR bound to these compounds (Fig. 2b, indicated by red arrows). Comparison of the ligated structures with unligated RamR revealed that the binding of cholic or chenodeoxycholic acid triggers uncoiling of the $\alpha 7b$ and $\alpha 8a$ helices (Fig. 2c), suggesting that RamR recognizes these compounds via an induced-fit mechanism. The binding position of cholic or chenodeoxycholic acid collides with helix $\alpha 7b$ (indicated by a red ribbon in Fig. 2c) of the unliganded RamR. Thus, the uncoiling of $\alpha 7b$ of RamR (indicated as a green ribbon in Fig. 2c) is required for the binding of these bile acid components. The $\alpha 8a$ helix is also uncoiled in ligated RamR, probably because of the uncoiling of $\alpha 7b$. These uncoiled forms of $\alpha 7b$ and $\alpha 8a$ were not observed in RamR structures bound to multiple other drugs (Fig. 2d) [12], indicating that this helix uncoiling is required for the recognition of bile acids. Although the five antimicrobial drugs were found to form $\pi$--$\pi$ interactions with Phe155 in a previous RamR structural study, this interaction does not occur with cholic or chenodeoxycholic acids (Fig. 3a and Supplementary Table S2). Instead of a $\pi$--$\pi$ interaction, both bile acids form four hydrogen bonds with the Tyr59, Thr85, Ser137 and Asp152 residues of RamR (Fig. 3b). This indicates that both cholic and chenodeoxycholic acids are recognized by RamR via the same mechanism. These interactions are different from the hydrogen bonds formed by the five antimicrobial drugs with other amino acid residues of RamR (Supplementary Table S2), indicating that the mechanism of bile acid recognition is separate to those of other antimicrobials drugs. The interaction of different sets of amino acid residues with each compound indicates that multiple compounds are recognized by the multisite-binding pocket of RamR.

**Reduction of RamR DNA-binding affinity by cholic acid and chenodeoxycholic acid.** Baucheron et al. proposed that RamR interacts with $P_\text{ramA}$ as a dimer of dimers [14]. We previously showed that the binding of multiple compounds to RamR reduces its DNA-binding affinity and results in the induction of $ramA$ expression [12]. The results of the present study suggest that the binding of cholic acid or chenodeoxycholic acid to RamR may also reduce its DNA-binding affinity. To quantify the effects of cholic and chenodeoxycholic acids on the interaction of RamR with its DNA-binding site, we performed SPR experiments using a purified RamR protein and 100-bp DNA fragment containing the RamR-binding site. RamR protein solution was passed over the DNA fragment immobilized on a sensor chip while in the presence or absence of cholic or chenodeoxycholic acid (up to 500 µM). Both bile acids inhibited the binding of RamR to the DNA, in a concentration-dependent manner (Fig. 4). The $IC_{50}$ values of cholic and chenodeoxycholic acids derived from the SPR data were 33.0 µM and 19.2 µM, respectively. In contrast, the much higher $IC_{50}$ value obtained with deoxycholic acid (318 µM) indicated a much weaker ability of this bile acid to reduce the RamR DNA-binding affinity (Fig. 4). This result is in good agreement with the qRT-PCR data, showing a lower activation of $ramA$ expression by deoxycholic acid than by cholic and chenodeoxycholic acids (Fig. 1c). Collectively, these results demonstrate that the interaction of cholic and chenodeoxycholic acids with RamR reduce its DNA-binding affinity and induce $ramA$ expression.

**Discussion**

RamA is known to be a major activator protein of the AcrAB-ToLC efflux system in S. Typhimurium, thus, it is involved in resistance to multiple drugs. Here, we report a regulatory mechanism for the expression of $ramA$ that involves the binding of its local RamR repressor protein to bile acids. The DNA-binding activity of RamR appears to be controlled by major bile acids, such as cholic or chenodeoxycholic acids.

The transcription of $acrAB$ and $tolC$ in *Salmonella* was previously known to be activated by bile [4]. However, the regulatory mechanisms involved in this activation have not been reported. The activation of $acrAB$ in *Salmonella* in response to bile seems to be independent of other regulatory proteins, such as MarRAB, Rob, RpoS or PhoP-PhoQ [14]. Since the AcrAB-ToLC efflux system exports bile salts and functions in both the bile resistance [15] and pathogenesis of *Salmonella* [16], it is important to understand the regulatory mechanism(s) of the $acrAB$ and $tolC$ genes in response to bile. We previously reported that bile induces AcrAB efflux pump expression in *Salmonella* through a specific regulatory protein, RamA, and, additionally, that cholic acid appears able to bind...
to this protein. More recently, we also identified a different induction mechanism of acrAB in response to bile, whereby the bile-mediated activation of the acrAB and tolC multidrug efflux genes occurs via transcriptional derepression of the ramA activator gene, likely via the RamR repressor protein controlling expression of ramA. Although the relative importance of both mechanisms has not yet been investigated, we suspect that these two different modes of regulation may coexist. A possible scenario is that bile first binds to and activates RamA and then, when the concentration of bile increases, proceeds to bind to RamR and stimulates RamA expression and the subsequent overproduction of the AcrAB-TolC efflux system. This scenario, however, requires further careful investigation.

The crystal structures of RamR in complexes with the components of bile reveal that both cholic and chenodeoxycholic acids form four hydrogen bonds with the Tyr59, Thr85, Ser137 and Asp152 residues of RamR, instead of π-π interaction with Phe155, an important residue for the recognition of multiple other drugs. Both cholic and chenodeoxycholic acids, but not deoxycholic acid, induce ramA expression in a RamR-dependent manner. We tried to crystalize RamR bound to deoxycholic acid but failed, probably because it lacks the 7α-hydroxyl group that is important in forming a hydrogen bond with Asp152 of RamR. Indeed, the inhibitory effect of deoxycholic acid on RamR-binding to the ramA promoter region is much weaker than that of cholic and chenodeoxycholic acids (Fig. 4). These acids inhibit interaction between RamR and the DNA fragment by approximately 90% or more at a concentration of 500 µM, whereas deoxycholic acid inhibits DNA binding by approximately 50% at the same concentration (Fig. 4b). This is consistent with the expression data, which show that deoxycholic acid induces ramA much less than cholic and chenodeoxycholic acids.

A similar approach to crystallizing a TetR-family regulator with bile acids has also been reported for CmeR, and its structures complexed with taurocholic or cholic acids have been determined at resolutions of 2.2 and 2.4 Å, respectively. These two elongated bile acids did not bind in the same orientation inside the CmeR tunnel but, in fact, lay antiparallel to each other. The crystal structure of CmeR complexed with glycerol suggests the presence of at least two distinct binding sites, while the CmeR-bile acid structures indicated that the large taurocholic and cholic acid molecules did not span the predicted binding sites, but instead bound to a distinct second site and left the glycerol-binding site unoccupied. Unlike the bile acid-binding site in CmeR, the RamR-binding site of cholic and chenodeoxycholic acids is located on the upper side of the protein at almost the same position as that of the five antimicrobial drugs berberine, crystal violet, dequalinium, ethidium bromide and rhodamine 6G (Fig. 2d).
The α7b and α8a helices were uncoiled in the structures of RamR in complex with cholic and chenodeoxycholic acids, but such uncoiling was not observed in RamR bound to the five antimicrobial drugs or CmeR in complex with taurocholic or cholic acids. Uncoiling of the α7b and α8a helices probably occurred due to the smaller size of the substrate-binding site of RamR compared to the relatively large cholic and chenodeoxycholic acid molecules.

In conclusion, we have extended our knowledge of the recognition of bile acids by RamR, a regulator of multidrug resistance in several enterobacterial pathogens. Cholic and chenodeoxycholic acids both form four hydrogen bounds with RamR, instead of the π–π interaction that is important for recognition of other drugs. These different recognition mechanisms highlight the wide substrate specificity of RamR, whereby the substrate-binding pocket accommodates a diverse array of ligands.

Methods

Bacterial strains, plasmids and growth conditions. The bacterial strains and the plasmids used in this study are listed in Supplementary Table 3. The S. enterica serovar Typhimurium strains were the wild-type strain ATCC14028s21 and its ΔramR mutant (which was produced as described previously)22. Bacterial strains were grown at 37 °C in Luria–Bertani broth supplemented, when appropriate, with ampicillin (100 µg/ml), kanamycin (25 µg/ml) or chloramphenicol (25 µg/ml).

In order to investigate the effects of the bile acids on ramA expression, 25.6 mg/ml of a crude ox bile extract purchased under the label ‘sodium choleate’ (Sigma-Aldrich, S9875), or 5 mM sodium cholate hydrate, sodium chenodeoxycholate or sodium deoxycholate monohydrate (C6445, C8261 and D5670, respectively, and all from Sigma) was added to the medium. Sodium choleate, whose precise composition was not determined, has previously been used in Salmonella gene expression experiments at concentrations of 3% (w/v), i.e., 30 mg/mL or higher8. We chose a concentration of 25.6 mg/mL, because it was the highest concentration that allowed the normal growth of the Salmonella strains used in this study.

Gene expression analysis by qRT-PCR. Bacteria were grown until mid-log phase (OD600 of 0.6) and harvested by centrifugation. Pelleted cells were stabilized with RNAprotect Bacteria Reagent (Qiagen) and stored at −80 °C until needed. Total RNA was extracted using an RNeasy Mini kit (Qiagen). Removal of residual genomic DNA was performed using a Turbo DNA-free kit (Ambion) and checked by negative PCR amplification of the chromosomal sequence. RNA integrity was verified by electrophoresis on a 1% agarose gel. Total RNA (1.5 µg) was reverse-transcribed using random hexamers and the Superscript III First Strand Synthesis System (Applied Biosystems). The expression level of each gene of interest was calculated as the average of the results from three independent cDNA samples. For each cDNA sample and each gene, qRT-PCR runs were performed in duplicated
well and the primers used for qRT-PCR are listed in Supplementary Table 4. The cycling conditions were: 95 °C for 5 min followed by 40 cycles of 95 °C for 10 s and then 60 °C for 15 s. After each run, amplification specificity and the absence of primer dimers were verified using a dissociation curve, acquired by heating the PCR products from 60 °C to 95 °C. The relative quantities of transcripts were determined using a standard curve and normalized against the geometric mean of three reference genes (gyrB, rrs and gyrA). A two-tailed Student's t test was used to assess significance using a p value < 0.05 as cutoff.

**Crystallography, data collection, and structure determination.** Purified RamR protein was prepared as described previously12. Co-crystals of RamR with cholic and chenodeoxycholic acids were grown from hanging drops at 25 °C using the vapor diffusion method. To form co-crystals, a 5-fold molar excess of cholic or chenodeoxycholic acids were added to 20 mg/ml RamR, then incubated overnight. The protein solution contained 20 mM sodium phosphate (pH 6.6), 75 mM NaCl and 2 mM DTT. The crystals grew to optimal size within 1 week and were cryoprotected using a solution containing 20% glycerol. Crystals were picked using LithoLoops (Protein Jet, 1.0–500 µl/vial). The crystal structures of bile-bound RamR were determined at 2.00 and 1.78 Å resolution by the molecular replacement method using the program MOLREP24 from the CCP4 software suite. The atomic coordinates of the ligand-free RamR (Protein Data Bank [PDB] code: 3VXV) were used as the search model. The model was refined with the CNSSO, REFMAC25 and COOT27 software and stereochemical quality was assessed with RAMPAGE28. Crystal parameters and refinement statistics are listed in Supplementary Table S1.

**SPR analysis.** The interaction between RamR and each substrate was analyzed by SPR spectroscopy with a Biacore T200 biosensor instrument (GE Healthcare). RamR was immobilized onto flow cells in a CM5 sensor chip using an amine-coupling method. Binding analyses were carried out at 25 °C and a flow rate of 30 µl/min. Cholic and chenodeoxycholic acids were passed over the RamR at several concentrations, as indicated. An empty flow-cell lacking immobilized protein was used as a reference. The inhibitory effects of cholic, chenodeoxycholic and deoxycholic acids on the interaction between RamR and P<sub>ramA</sub> were also analyzed by SPR spectroscopy. A 3′-biotinylated 100-bp DNA fragment of the ramR–ramA intergenic region, as well as a control 100-bp control fragment of the gyrB gene, were each immobilized onto flow cells in a NeutrAvidin (Thermo Scientific)-coated CM5 sensor chip (GE Healthcare). Analyses were performed at 25 °C and at a flow rate of 30 µl/min. The purified RamR protein was diluted in running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% v/v surfactant P20), incubated with 1.0–500 µM of cholic, chenodeoxycholic or deoxycholic acids, then injected onto the sensor surface in two replicates for 2 min. Dissociation was also recorded for 2 min. Calculations of affinity constants were performed using BIAevaluation software (GE Healthcare).

**Accession codes.** Protein Data Bank: coordinates have been deposited under accession codes 6IE8 and 6IE9 for the RamR structures co-crystallized with cholic and chenodeoxycholic acids, respectively.

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**Acknowledgements**

Crystallographic data were collected at Osaka University’s beamline BL44XU in Spring-8. This work is supported in part by Grant-in-Aid for Young Scientist, Scientific Research, Challenging Research (Exploratory) from Japan Society for the Promotion of Science, JSPS (Grant Numbers 26713004, 17H03983, and 18K19451), by the Center of Innovation Program from Japan Science and Technology Agency, JST, by Japan Agency for Medical Research and Development (AMED), by the Waksman Foundation of Japan, by Network Joint Research Center for Materials and Devices, by Dynamic Alliance for Open Innovation Bridging Human, Environment and Materials, and by the Institut National pour la Recherche Agronomique (INRA) as an INRA-JSPS joint research project. It was also supported by the French Région Centre (Grant Number 2008 00036085) and partly by the European Union with the European Regional Development Fund (Grant Number 1634–32245).

**Author Contributions**

S.Y., R.N. and K.S. performed the crystallographic analysis. S.Y., S.B., E.G., B.D., A.C. and K.N. performed the molecular biological and biochemical analyses. K.N. designed the study. All authors discussed the data and wrote the manuscript. K.N. supervised all the work.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-36025-8.

**Competing Interests:** The authors declare no competing interests.

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