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Aromatic Acid Metabolites of Escherichia coli K-12 Can Induce the marRAB Operon

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Aromatic Acid Metabolites of *Escherichia coli* K-12 Can Induce the marRAB Operon

Post-Print

Abstract: MarR is a key regulator of the marRAB operon involved in antibiotic resistance and solvent stress tolerance in *Escherichia coli*. We show that two metabolic intermediates, 2,3-dihydroxybenzoate and anthranilate, involved in enterobactin and tryptophan biosynthesis, respectively, can activate marRAB transcription. We also found that a third intermediate involved in ubiquinone biosynthesis, 4-hydroxybenzoate, activates marRAB transcription in the absence of TolC. Of the three, however, only 2,3-dihydroxybenzoate directly binds MarR and affects its activity.

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A central regulator of intrinsic antibiotic resistance and organic acid tolerance in *Escherichia coli* and related enteric bacteria is MarR, a negative autoregulator of the *marRAB* operon (2, 4, 7–8, 10, 17–19). This protein is a canonical member of a family of transcriptional repressors commonly associated with regulating the expression of multidrug efflux systems, stress response systems, metabolic pathways, and virulence factors (reference 24 and references therein). A common theme among MarR family regulators is the ability to bind structurally disparate anionic lipophilic compounds. In the case of MarR, numerous chemical compounds, such as benzoate, salicylate, 2,4-dinitrophenol, menadione, and plumbagin, have been shown to modulate its activity both *in vivo* and *in vitro* (1, 3, 9, 19, 22). Additionally, MarR activity is affected through protein-protein interactions with enzymes such as transketolase A and DNA gyrase subunit A (11–12).

Recently, Rosner and Martin found that in an *E. coli tolC* mutant, the *marRAB* promoter was upregulated approximately 2-fold (21). They concluded from these results that upon the loss of TolC-dependent excretory capacity, cells accumulate metabolic intermediates capable of inducing the *mar* system. Intrigued by this hypothesis, we examined a number of metabolic pathways and hypothesized that those involved in the superpathway of chorismate may be potential effectors of MarR given their chemical similarity to its known ligands. In this work, we show that three such intermediates in aromatic amino acid biosynthesis activate *marRAB* transcription *in vivo* when supplied endogenously and that one, 2,3-dihydroxybenzoate, directly binds to MarR *in vitro*.

Aromatic metabolic intermediates activate the *marRAB* operon *in vivo*. Of the several known inducers of *marRAB* expression, the best characterized is salicylate, a weak aromatic acid (1, 3, 9, 19, 22). Specifically, salicylate has been shown to directly bind MarR (3, 19). If metabolic intermediates are potential ligands for MarR, we surmised that they may have chemical structures similar to that of the canonical inducer salicylate. Among the vast number of metabolic intermediates in *E. coli* metabolism, those involved in the superpathway of chorismate appeared to be the most promising (14).

Examination of this superpathway in *E. coli* K-12 yielded four compounds that were structurally similar to salicylate (Fig. 1A). Our basis for assessing similarity was the presence of a carboxylate group with adjacent hydroxyl or amine groups on the benzyl ring. Four putative compounds were identified: 4-hydroxybenzoate (HBA), an intermediate in ubiquinone-8 biosynthesis; 2,3-dihydroxybenzoate (DHB), an intermediate in enterobactin biosynthesis; anthranilate (ANT), an intermediate in tryptophan biosynthesis; and 4-aminobenzoate (PABA), an intermediate in tetrahydrofolate biosynthesis.

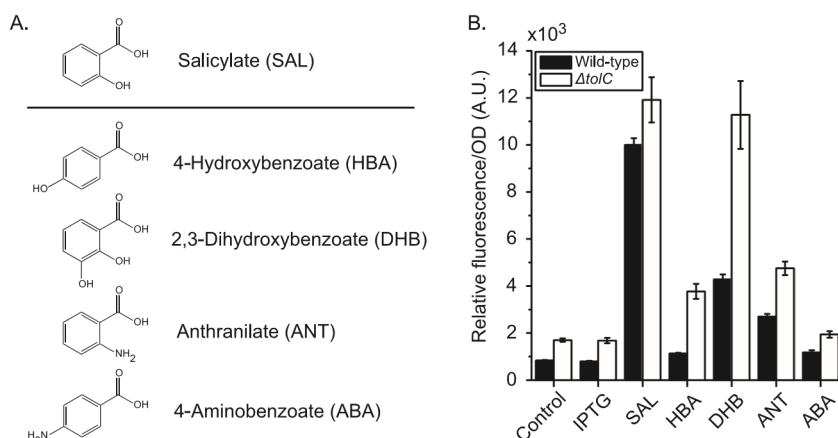


FIG. 1. Activation of the *marRAB* operon by aromatic acid metabolites in the superpathway of chorismate. (A) Chemical structure of metabolites selected based on their similarity to salicylate. (B) Observed transcriptional activation of the *marR-yfp* promoter fusion in the presence of 5 mM concentrations of indicated inducers in wild-type (CR700) and $\Delta tolC$ mutant (CR703) backgrounds. Salicylate and IPTG served as positive and negative controls for activation, respectively. Cells were grown overnight in MOPS (morpholinepropanesulfonic acid) minimal medium (20 mM glucose, 0.2% Casamino Acids, pH 7.2) and subcultured 1:200 in fresh medium. Following dilution, 450 l of culture was transferred to deep 96-well plates and grown at 37°C with aeration at 1,000 rpm to an OD of 0.5. At this time, 100 l of medium containing dissolved inducer was added. Growth was continued for an additional 2 h prior to fluorescence and optical density measurements made with a Tecan Safire2 microplate reader. An expanded description of the experimental procedures is provided in the supplementary material. A.U., arbitrary units.

To test whether these compounds were inducers of the *marRAB* operon, we used a chromosomal, single-copy transcriptional fusion of the *marRAB* promoter to the fast-folding yellow fluorescent protein (YFP) variant *Venus* to monitor gene expression in the presence of the four potential inducers (20). For comparison, we also explored the abilities of these chemicals to activate expression in a *tolC* null mutant. Of the four compounds, we found that only DHB and ANT activate the *marRAB* promoters in wild-type cells (Fig. 1B). We also found that HBA could activate the *marRAB* promoter in a *tolC* null mutant. Interestingly, PABA failed to activate the *marRAB* promoter despite its chemical similarity to salicylate.

MarR binds 2,3-dihydroxybenzoate. We next tested whether MarR binds directly to DHB, ANT, and HBA. To measure binding, we employed isothermal titration calorimetry (ITC) using purified MarR (Fig. 2). Consistent with previous measurements, we found that the affinity of MarR for salicylate was at a K_d (dissociation constant) of 0.9 mM, well within the ranges reported using other methods (1, 19). Binding was not observed with isopropyl--D-thiogalactopyranoside (IPTG), which served as our negative control. We also found that MarR bound DHB with a K_d of 0.5 mM. However, we found that

ANT did not bind to MarR even though it was capable of activating the *marRAB* promoter. Similarly, we found that HBA, which activates the *marRAB* promoter only in the absence of *tolC*, also did not bind to MarR (data not shown). This suggests that these two compounds may indirectly regulate *marRAB* promoter activity.

We note that despite the fact that MarR had similar affinities for salicylate and DHB, salicylate has a greater effect than DHB on *marRAB* activation *in vivo*. To explain these results, we imagine that DHB is likely being metabolized by the cell. Consistent with this hypothesis, we observed increased levels of DHB-dependent *marRAB* promoter activation in a *tolC* mutant background (Fig. 1B), suggesting that loss of the excretory function of TolC may lead to a buildup of DHB, most likely by preventing the efflux of the downstream metabolite, enterobactin (5).

MarR activity is modulated *in vitro* by 2,3-dihydroxybenzoate. As MarR binds to DHB, we next tested whether DHB affects MarR binding to the *marRAB* promoter region. To determine the loss of MarR binding activity in the presence of DHB, we employed electrophoretic mobility shift assays using purified MarR and a 150-bp region of the *marRAB* promoter (see the

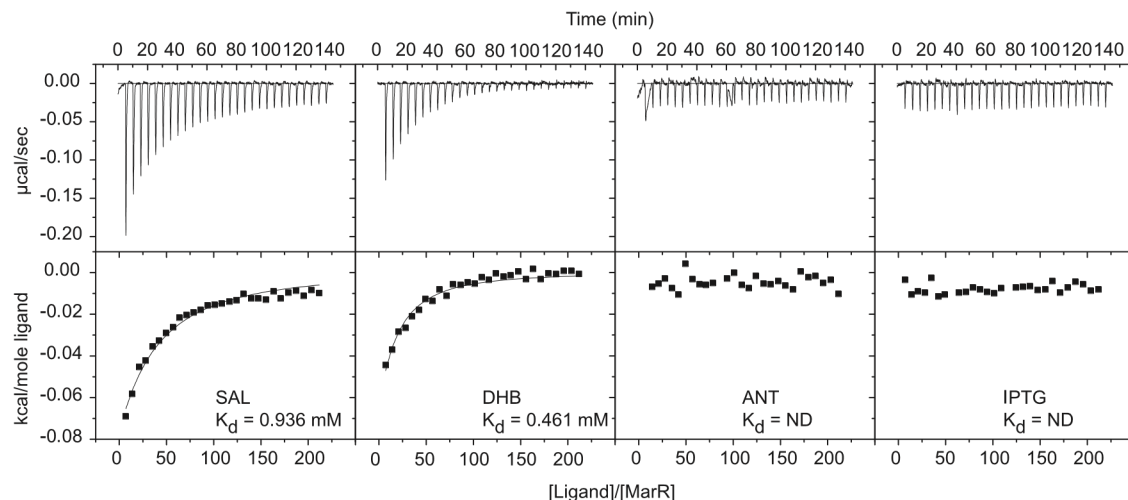


FIG. 2. MarR binding of aromatic acid inducers as determined by isothermal titration calorimetry (ITC). Experiments were conducted using a VP-ITC calorimeter (MicroCal) with 1.4 ml MarR at 10 M and ligands at a concentration of 10 mM, both in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4). In the cases of ANT and HBA, higher concentrations of ligand were also tested though again no binding was observed (data not shown). Titration reactions were performed with 28 injections, all 10 l in volume, with constant stirring at 300 rpm at 25°C. Data acquisition and binding coefficients were determined with the Origin-based MicroCal analysis software.

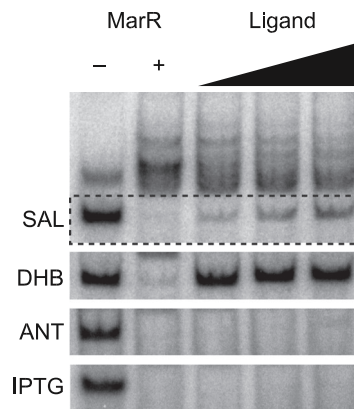


FIG. 3. MarR DNA binding activity in the presence of aromatic acid metabolites as determined by electromobility shift assays. Binding reaction mixtures consisted of 20 ng of purified MarR and 5 ng of a radiolabeled, 150-bp fragment of the *marRAB* promoter containing two MarR operator sites. The ligands salicylate (SAL), 2,3-dihydroxybenzoate (DHB), anthranilate (ANT), and IPTG were supplied at increasing concentrations of 2.5 mM, 5 mM, and 10 mM to the binding reaction mixtures. Reactions were displayed on 5% acrylamide, 0.5 Tris-borate-EDTA (TBE)-buffered gels. Loss of MarR DNA binding activity was monitored by the emergence of free DNA in the presence of these ligands. Salicylate and IPTG served as positive and negative controls, respectively. A detailed description of the methods is provided in the supplemental material.

supplemental material for details). Using salicylate as our positive control and IPTG as our negative control, we found that only DHB directly affected MarR activity (Fig. 3). Interestingly, we observed significantly more unbound DNA in the presence of DHB than salicylate, even though MarR has similar binding affinities for the two. We also tested whether ANT affected MarR activity and found that it did not, consistent with our ITC experiments.

Disruptions in enterobactin and tryptophan biosynthesis affect *marRAB* promoter activity in *tolC* mutants. Our previous results have demonstrated that when added exogenously, DHB and ANT activate the *marRAB* promoter *in vivo*. To better correlate the metabolite effector hypothesis of Rosner and Martin to these observations, we disrupted the enzymatic steps in enterobactin and tryptophan metabolism that block either the synthesis or utilization of DHB or ANT. Specifically, mutants lacking the enterobactin synthesis pathway (*entCEBAH*) or deficient in 2,3-dihydro-2,3-dihydroxybenzoate (DDHB) dehydrogenase (*entA*), enterobactin synthase (*entF*), anthranilate synthase (*trpE*), and phosphoribosyl transferase (the C-terminal region of *trpD*) activities were constructed in otherwise wildtype and *tolC* mutant strains and tested for alterations in *marRAB* promoter activity.

In *TolC* backgrounds, we observed no significant changes in *marRAB* promoter activity in any of the mutants, presumably due to the ability of these cells to readily excrete accumulated intermediates (data not shown). However, when we repeated these experiments in the absence of *TolC*, we found that the *marRAB* promoter

was less active in mutants unable to synthesize enterobactin from chorismate ($1,559 \pm 170$ relative fluorescence units per optical density unit [RFU/OD] [*tolC* mutant] versus $1,387 \pm 195$ RFU/OD [Δ *tolC* Δ *entCEBAH* mutant], $P = 0.001$). However, when we attempted to accumulate the DHB in the cell by blocking conversion of DHB to enterobactin, we observed a decrease in *marRAB* promoter activity as opposed to an expected increase ($1,559 \pm 170$ RFU/OD [Δ *tolC* mutant] versus $1,491 \pm 78$ RFU/OD [Δ *tolC* Δ *entF* mutant], $P = 0.04$). While statistically significant, the effect is minor and likely not physiologically significant. We suspect that this mutant (*tolC entF*) does not accumulate significant amounts of DHB. Interestingly, we observed a significant increase in *marRAB* promoter activity when we blocked the conversion of DDHB to DHB ($1,559 \pm 170$ RFU/OD [Δ *tolC* mutant] versus $1,737 \pm 133$ RFU/OD [Δ *tolC* Δ *entA* mutant], $P = 0.0001$). Given the similar chemical structures of DDHB and DHB, differing only by a hydrated 2,3 carbon-carbon bond on the benzyl ring, we suspect that DDHB may accumulate in this mutant and serve as an alternate activator of MarR. Collectively, these results suggest that enterobactin intermediates are physiological activators of the *marRAB* operon.

We also investigated the effects of tryptophan biosynthesis on *marRAB* expression in the absence of TolC. We found that mutants unable to convert ANT to tryptophan did not exhibit any significant changes in *marRAB* promoter activity (data not shown). Interestingly, in the absence of anthranilate synthase, and therefore the ability to synthesize ANT, we observed a statistically significant increase in the *marRAB* promoter activity ($1,516 \pm 68$ RFU/OD [Δ *tolC* mutant] versus $1,702 \pm 173$ RFU/OD [*tolC trpE* mutant], $P < 0.00002$). We suspect that blocking the initial step of tryptophan biosynthesis may redirect metabolic fluxes to other pathways where the metabolite intermediates induce *marRAB* expression. Taken together, these results indicate that disruption of tryptophan biosynthesis affects *marRAB* expression, although they suggest that tryptophan intermediates do not contribute directly to the *tolC* phenotype. Moreover, the effect is not direct nor is the mechanism clear. We were not entirely surprised by this result, as we found that ANT does not directly bind MarR and affect its activity. Rather, the effect appears to be indirect.

Conclusions. A number of studies have shown that some metabolic intermediates are inducers and substrates for various efflux pumps (6, 13, 15–16, 23). Likely, these mechanisms prevent the buildup of toxic metabolic intermediates. In support of this model, our results demonstrate that MarR directly binds one such intermediate, 2,3-dihydroxybenzoate, that is involved in the biosynthesis of enterobactin, itself a substrate for TolC (5). Whether DHB is a MarR effector under physiological conditions, however, is still unknown.

While these results suggest that enterobactin biosynthesis contributes to the increase in *marRAB* expression in the absence of TolC, they do not explain the phenotype completely. Due to the magnitude of the changes in *marRAB*

expression caused by mutations in the enterobactin pathway, we suspect that the true source of increased activation is likely a combination of many intracellular metabolites, as proposed by Rosner and Martin (21).

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