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Characterization of the *Escherichia coli* AaeAB Efflux Pump: a Metabolic Relief Valve?

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Treatment of *Escherichia coli* with *p*-hydroxybenzoic acid (pHBA) resulted in upregulation of *yhcP*, encoding a protein of the putative efflux protein family. Also upregulated were the adjacent genes *yhcQ*, encoding a protein of the membrane fusion protein family, and *yhcR*, encoding a small protein without a known or suggested function. The function of the upstream, divergently transcribed gene *yhcS*, encoding a regulatory protein of the LysR family, in regulating expression of *yhcRQP* was shown. Furthermore, it was demonstrated that several aromatic carboxylic acid compounds serve as inducers of *yhcRQP* expression. The efflux function encoded by *yhcP* was proven by the hypersensitivity to pHBA of a *yhcP* mutant strain. A *yhcS* mutant strain was also hypersensitive to pHBA. Expression of *yhcQ* and *yhcP* was necessary and sufficient for suppression of the pHBA hypersensitivity of the *yhcS* mutant. Only a few aromatic carboxylic acids of hundreds of diverse compounds tested were defined as substrates of the YhcQP efflux pump. Thus, we propose renaming *yhcS*, *yhcR*, *yhcQ*, and *yhcP*, to reflect their role in aromatic carboxylic acid efflux, to *aaeR*, *aaeX*, *aaeA*, and *aaeB*, respectively. The role of pHBA in normal *E. coli* metabolism and the highly regulated expression of the AaeAB efflux system suggests that the physiological role may be as a "metabolic relief valve" to alleviate toxic effects of imbalanced metabolism.

A fundamental assumption underlying gene expression analyses is that bacterial cells typically respond to externally imposed stresses by inducing expression of defense proteins that combat the specific stress condition. In response to treatment with chemicals and antibiotics, such defense mechanisms often include elevated expression of energy-dependent efflux transport systems. For example, the primary multidrug efflux pump in *Escherichia coli*, AcrAB-TolC, is upregulated via several mechanisms in response to treatments with diverse compounds such as salicylic acid (22), methylviologen (34), or bile salts (33). A wide range of compounds are substrates for the AcrAB-TolC efflux system (27), so that increased expression of the efflux pump leads to decreased intracellular concentrations and thus higher tolerance to the externally added compounds.

Efflux systems range from the broad substrate specificity of multidrug efflux pumps with complex regulation to those with very narrow substrate specificity and specific regulation. Examples of the latter class are found in metal ion efflux systems. For instance, the CusCFBA complex exports copper and silver ions from *E. coli* cells (13). Expression of the CusCFBA efflux system is regulated by a two-component regulatory system in response to the presence of its substrates, copper or silver ions (12, 25). Thus, in general, treatment with a chemical may trigger upregulation of efflux pumps for which that chemical is a substrate. Accordingly, as data from genome-wide analyses are increasingly being generated, clues to undefined efflux gene function may be found from transcriptional or translational responses to chemicals.

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Both the AcrAB-TolC and the CusCFBA transporters are examples of multicomponent efflux systems in gram-negative bacteria. The inner membrane components, such as AcrB or CusA, are the energy-dependent transport proteins. Both AcrB and CusA are members of the resistance/nodulation/cell division (RND) superfamily of proteins (46), which catalyze substrate efflux by an H^+ antiport mechanism. However, the efflux pump proteins in other multicomponent efflux systems may come from several protein families (38). The periplasmic proteins in the multicomponent efflux systems, such as AcrA or CusB, are members of the membrane fusion protein (MFP) family that are essential for efflux activities of the corresponding transporters (10). Proteins from the outer membrane factor (OMF) family (28), such as TolC or CusC, span the outer membrane and periplasmic space connecting with the inner membrane efflux pumps and provide a channel for efflux of substrate molecules from the cytoplasm to the outside of the cell (18). The AcrAB-TolC transporter is a tripartite system, as are numerous other efflux transporters in gram-negative bacteria. Interestingly, CusCFBA is an example of a rare four-component transport system. CusF is a small periplasmic copper binding protein that increases the efficiency of copper efflux (13).

An informatics analysis conducted by Harley and Saier identified a family of putative efflux transport (PET) proteins in bacteria, yeast, and plants (15). The proteins of the PET family in gram-negative bacteria have 12 predicted transmembrane segments, indicating membrane localization. In many cases, genes for PET family proteins are found adjacent to genes encoding MFP family proteins that function in efflux transport. However, experimental evidence of PET function has not been published, nor have any predictions for substrates been made. During genome-wide analysis of transcriptional responses of *E.*

TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype or characteristic	Reference or source
Strains		
DE112	*rpsL galK2* Δ*lac74 tolC*::mini-Tn*10*	49
DPD1675	*ilvB2101 ara thi* Δ(*proAB-lac*) *tolC*::mini-Tn*10*	50
DPD1818	*tolC*::mini-Tn*10* of MG1655	This work
DPD2410	*yhcS*::TN⟨Kan⟩ of DH5αE	This work
DPD2433	*yhcS*::TN⟨Kan⟩ of MG1655	This work
DPD2435	*yhcS*::TN⟨Kan⟩ *tolC*::mini-Tn*10* of MG1655	This work
DPD2443	*yhc*P::TN⟨Kan⟩ of DH5αE	This work
DPD2444	*yhc*P::TN⟨Kan⟩ of MG1655	This work
MG1655	F^- *rph-1 fnr*	14
Plasmids		
pDEW215	*yciG-luxCDABE*	48
pDEW558	*lysU-luxCDABE*	This work
pDEW655	*yhcRQP-luxCDABE*	This work
pKK223-3		Pharmacia
pDEW659	*yhcP* in pKK223-3	This work
pTrcHis2TOPO/lacZ		Invitrogen
pDEW668	*yhcRQP* in pTrcHis2TOPO	This work
pDEW673	*yhcQP* in pTrcHis2TOPO	This work
pDEW675	*yhcRQ* in pTrcHis2TOPO	This work

coli treated with *p*-hydroxybenzoic acid (pHBA), a compound for which microbial bioproduction is being developed (2, 23, 31), we noted dramatically elevated expression of a gene encoding a PET family protein and two adjacent genes. Thus, the role of these proteins in pHBA efflux as a defense mechanism triggered by excess pHBA was investigated.

MATERIALS AND METHODS

***E. coli* strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. *E. coli* MG1655 (14) was used for most experiments. Subsequently, it was discovered to be an *fnr* mutant (T. K. Van Dyk, unpublished data), as are some other lab strains of MG1655 (43). We do not, however, expect that the *fnr* mutation would affect the results reported here. A previously constructed and mapped large collection of transposon insertion mutations (39) in *E. coli* strain DH5αE contains EZ::TN⟨Kan-1⟩ insertions in *yhcS* and *yhcP*. The locations of these insertions were verified by PCRs with gene-specific and transposon-specific primers. P1clr100Cm-mediated transduction (24) was used to construct derivatives of *E. coli* strain MG1655 with *yhcS*::TN⟨Kan⟩, *yhcP*::TN⟨Kan⟩, or *tolC*::mini-Tn*10*.

Plasmids pDEW215, pDEW558, and pDEW655 each carry functional *Photorhabdus luminescens luxCDABE* gene fusions from the LuxA collection of *E. coli* gene fusions (51). Plasmid pDEW215 carrying a *yciG-luxCDABE* gene fusion has been previously described (48). Plasmids pDEW558 (lux-a.pk033.e5) and pDEW655 (lux-a.pk035.c7) contain *E. coli* chromosomal segments between nucleotides 4350990 and 4353107 and nucleotides 3385829 and 3386761 (4), respectively. Accordingly, light production from strains carrying plasmid pDEW558, which contains the *lysU* promoter, reports *lysU* promoter activity. Likewise, plasmid pDEW655, which contains the promoter region of the putative *yhcRQP* operon, provides a reporter of *yhcRQP* expression.

Plasmid pDEW659 carries the *yhcP* gene in a moderate-copy-number vector. PCR amplification used primers 5′GTTAGCAAGCTTTTAACTATCGGTCAACGCAT3′ and 5′AGCAGTGAATTCATGGGTATTTTCTCCATTGC3′ with HindIII or EcoRI sites for directional cloning. The PCR product and plasmid pKK223-3 at an approximate molar ratio of 2:1 were digested with EcoRI and HindIII, purified, and ligated. Following ligation, digestion with PstI and SmaI was used to linearize vector without insert DNA. The DNA from this selection digestion was used for transformation of *E. coli* strain JM105. One plasmid with an insert of the correct size was retained.

Plasmids pDEW668, pDEW673, and pDEW675 carry all or part of the *yhcRQP* operon expressed from the *trc* promoter in a high-copy-number plasmid. The PCR primers were designed such that when the amplified DNA was cloned into the pTrcHis2TOPO vector (Invitrogen), N-terminal fusion proteins or C-terminal fusion proteins would not be formed and thus the native proteins would be expressed. Also, an EcoRI site incorporated into one of each set of primers was used to determine orientation of the inserted DNA. The primers to amplify the entire *yhcRQP* operon were 5′GAAGTTAGCAAGCTTTTAACTATCGGTCAACGCATG3′ and 5′ACAGGAGAATGAATTCATGCCCTTCTCTGCGGCGAC3′. Those used to amplify *yhcQP* genes were 5′TTAACTATCGGTCAACGCATGTT 3′ and 5′ACAGGAGAATGAATTCGTGAAAACACTAATAAGAAA 3′. The *yhcRQ* genes were obtained by using the primers 5′TTAACCAAACTCACGCAGGC3′ and 5′ACAGGAGAATGAATTCATGCCCTTCTCTGCGGCGAC3′. The purified PCR products were cloned into pTrcHis2TOPO according to the protocol supplied by the vendor. After transformation of *E. coli* strain TOP10 (Invitrogen), plasmid DNA from individual transformants was digested with EcoRI. Plasmids with the proper size and orientation of inserted DNA were retained and verified by DNA sequence analysis.

Media and chemicals. All reagents and materials used for the growth and maintenance of bacterial cells were obtained from Aldrich Chemicals (Milwaukee, Wis.), Difco Laboratories (Detroit, Mich.), Gibco/BRL (Gaithersburg, Md.), or Sigma Chemical Company (St. Louis, Mo.). Either rich Luria-Bertani (LB) medium (24) or defined Vogel-Bonner medium (9) with 0.4% glucose as a carbon source was used. When necessary, ampicillin was added for plasmid maintenance at 25 μg/ml in defined medium or at 100 μg/ml in rich medium.

DNA microarray. DNA microarray analysis (29) was used to assess transcriptional alterations of essentially all *E. coli* genes following treatment with pHBA. *E. coli* strain DE112 was grown in Vogel-Bonner minimal medium to an optical density at 600 nm of 0.2. At this point the culture was split in two flasks, and pHBA in the acid form was added to one flask from a stock solution in ethanol to achieve a final pHBA concentration of 25 mM. An equivalent volume of ethanol without pHBA was added to the other flask. Approximately 58% growth inhibition resulted from the pHBA treatment under these conditions. Cells were harvested from the control and treated flasks at 30 and 60 min after pHBA addition and were used for RNA isolation. RNA isolation, labeling, and hybridization and data acquisition were done as previously described (42, 52).

Luminometry. *E. coli* strain DPD1675, MG1655, or DPD2433 carrying plasmid pDEW655 was used to measure the bioluminescent response to various chemicals. Aliquots (50 μl) of actively growing cultures at 37°C in LB medium were added to 50 μl of LB medium (pH 7) containing either pHBA, 1-naphthoate, benzoate, or salicylate in the wells of a white microplate. The sodium salt form of the chemicals was used so that the initial pH of the medium remained neutral. Twofold dilutions of each chemical were tested at seven final concentrations in duplicate. Bioluminescence was measured as relative light units with a Luminoskan microplate luminometer (LabSystems) at 37°C in the kinetic mode immediately after the cell cultures were added.

To test responses to weak acids, *E. coli* strain DPD1675 carrying either pDEW215, pDEW558, or pDEW655 was grown overnight in Vogel-Bonner medium supplemented with L-proline, L-lysine, uracil, and 25 μg of ampicillin per ml. The overnight cultures were diluted into the same medium except without

ampicillin and incubated at 37°C until they were in mid-exponential growth. Aliquots (50 μl) of these actively growing cultures were added to 50 μl of the same medium (pH 7) containing various concentrations of sodium acetate or sodium salicylate in duplicate in the wells of a white microplate. Immediately after addition of the cell culture, the bioluminescence was quantitated in a Luminoskan microplate luminometer at 37°C in the kinetic mode.

MIC determination and zones of growth inhibition. For tests without tetrazolium violet, MICs were determined by visually assessing turbidity. Dilution series, 3:4 or 1:2, were used in duplicate to test a range of chemical concentrations in a 100-μl final volume in microplates. The inoculum was 50 μl of a 1:500 dilution in Vogel-Bonner medium of overnight cultures of each of the strains grown in the same medium, except with addition of 25 μg of ampicillin per ml for plasmid-containing strains. For this test, the MIC was defined as the lowest concentration that resulted in complete lack of turbidity after incubation for 21 h at 37°C.

For screening large numbers of chemicals, 0.01% tetrazolium violet was added as an indicator of viability of *E. coli* MG1655 (*yhcP*$^+$) and DPD2444 (*yhcP*) in Vogel-Bonner medium. Twofold dilution series of each chemical were tested in a 100-μl final volume. The inoculum was 50 μl of an overnight culture in Vogel-Bonner medium that had been previously diluted 500-fold into fresh medium. Following incubation at 37°C without shaking for 16 to 18 h, the purple color was visually scored. A score of 4+ indicated full purple color equivalent to that of a no-chemical control. A score of 3+, 2+, or 1+ indicated decreasing color, while a score of 0 indicated the complete absence of color. The MIC was defined as the lowest tested concentration that gave complete absence of color. The rank of difference between strains was defined as the score of pigment color for *E. coli* MG1655 in wells containing the concentration of a given chemical at the MIC for DPD2444. When MG1655 had no color, the rank was 0. When MG1655 was scored 1+, the rank was 1; when it was scored 2+, the rank was 2; etc.

Tests of the zone of growth inhibition on Vogel-Bonner plates were done with 0.1 ml of overnight cultures plated with 2.5 ml of Vogel-Bonner soft agar. The zone of growth inhibition surrounding a disk containing 80 μmol of the pHBA sodium salt was measured after 24 h of incubation at 37°C.

RESULTS

DNA microarray gene expression profiling of pHBA-treated *E. coli*. The alterations in the *E. coli* gene transcriptional profile upon exposure to the pHBA were characterized by using DNA microarray technology. Following 60 min of exposure to 25 mM pHBA, we observed 105 genes for which expression was upregulated threefold or greater. Of these, 23 genes were upregulated 10-fold or more. This group of highly upregulated genes included a putative operon, *yhcRQP*. The signals hybridizing to the *yhcR*, *yhcQ*, and *yhcP* spotted DNAs were increased 10-, 22-, and 12-fold, respectively.

The *E. coli yhcP* gene, encoding a PET family protein, is predicted (45) to be cotranscribed with two nearby genes, *yhcQ* and *yhcR*. The order of genes in this putative operon is *yhcRQP*. The product of *yhcQ* likely functions in cellular efflux because it is an MFP family protein (15). The third gene of the putative operon, *yhcR*, encodes a protein for which no prediction of function has been made. The observed coregulation in response to pHBA treatment is consistent with the predicted operon structure, and thus these three genes will be referred to as the *yhcRQP* operon.

Regulation of *yhcRQP* expression by YhcS. The *yhcS* gene of *E. coli* encodes a member of the LysR family of positive-acting regulatory molecules (37). This gene is located immediately adjacent to and is divergently transcribed from the *yhcRQP* operon. The possibility that YhcS controls expression of *yhcRQP* was tested by using a *yhcS* null mutation and a plasmid carrying a *yhcRQP-luxCDABE* gene fusion. Table 2 shows the bioluminescent response a *yhcS*::TN⟨Kan⟩ mutant and an otherwise isogenic *yhcS*$^+$ strain after 30 min of pHBA exposure.

TABLE 2. Bioluminescence response of the *yhcRQP-luxCDABE* gene fusion to pHBA[a]

[pHBA], mM	Relative light units	
	yhcS$^+$	*yhcS*
0	0.631	0.815
1.6	6.72	1.02
3.1	10.2	1.16
6.2	16.2	1.42
12.5	30.8	1.82
25	66.6	1.59
50	91.7	0.614
100	0.437	0.045

[a] *E. coli* strains MG1655 carrying pDEW655 and DPD2433 carrying pDEW655 were used. Log-phase cultures in LB medium were treated with the indicated concentrations of pHBA for 30 min.

Relatively high concentrations of pHBA were used because the uncharged acid form, which is the moiety that diffuses into the cell, is a minor component at neutral pH.

In the *yhcS*$^+$ strain, elevated expression of the *yhcRQP* operon was induced at all concentrations of pHBA tested except 100 mM, which was likely sufficiently toxic to interfere with the energy and reducing-power production required for the bioluminescence reactions (47). At sublethal concentrations of pHBA, the level of gene expression increased with increasing concentration of pHBA, up to a 145-fold increase at 50 mM pHBA. This observed upregulation of the *yhcRQP-luxCDABE* gene fusion thus confirmed the DNA microarray result. In contrast, the *yhcS*::TN⟨Kan⟩ mutation almost completely eliminated the increased expression induced by pHBA treatment. Accordingly, functional YhcS is required for upregulation of *yhcRQP* expression in response to pHBA addition. These results compellingly imply that YhcS functions, in analogy to other LysR family members, as a positive transcription factor for the *yhcRQP* operon.

Structure-activity relationships for YhcS activation. Further characterization of the signals that trigger activation of YhcS was done by using *E. coli yhcS*$^+$ strains, DPD1675 and MG1655, carrying the *yhcRQP-luxCDABE* gene fusion. In addition to pHBA, several aromatic weak acid molecules also activated expression. In the DPD1675 (*tolC*) host strain, 25 mM pHBA resulted in 300-fold-elevated bioluminescence. In this strain, 1-naphthoate elevated *yhcRQP* expression by 130-fold. In the MG1655 host strain (*tolC*$^+$), 12.5 mM sodium benzoate and 6.2 mM sodium salicylate resulted in 12- and 77-fold increases in bioluminescence, respectively. Thus, the inducing molecules include pHBA, salicylate, benzoate, and 1-naphthoate.

Compounds tested that did not induce expression were operationally defined as those for which there resulted less than a threefold increase in light production from *E. coli* strains carrying the *yhcRQP-luxCDABE* gene fusion. Compounds unrelated in structure to the known inducing molecules did not induce expression. Those tested were ethanol, limonene, NaCl, polymyxin sulfate, benzalkonium chloride, gramicidin S, and sodium dodecyl sulfate (data not shown). In addition, several compounds related in structure to the inducing molecules were not inducers, including acetate, propionate, methyl paraben, 2-biphenylcarboxylate, and L-tyrosine (see Fig. 2A; other data

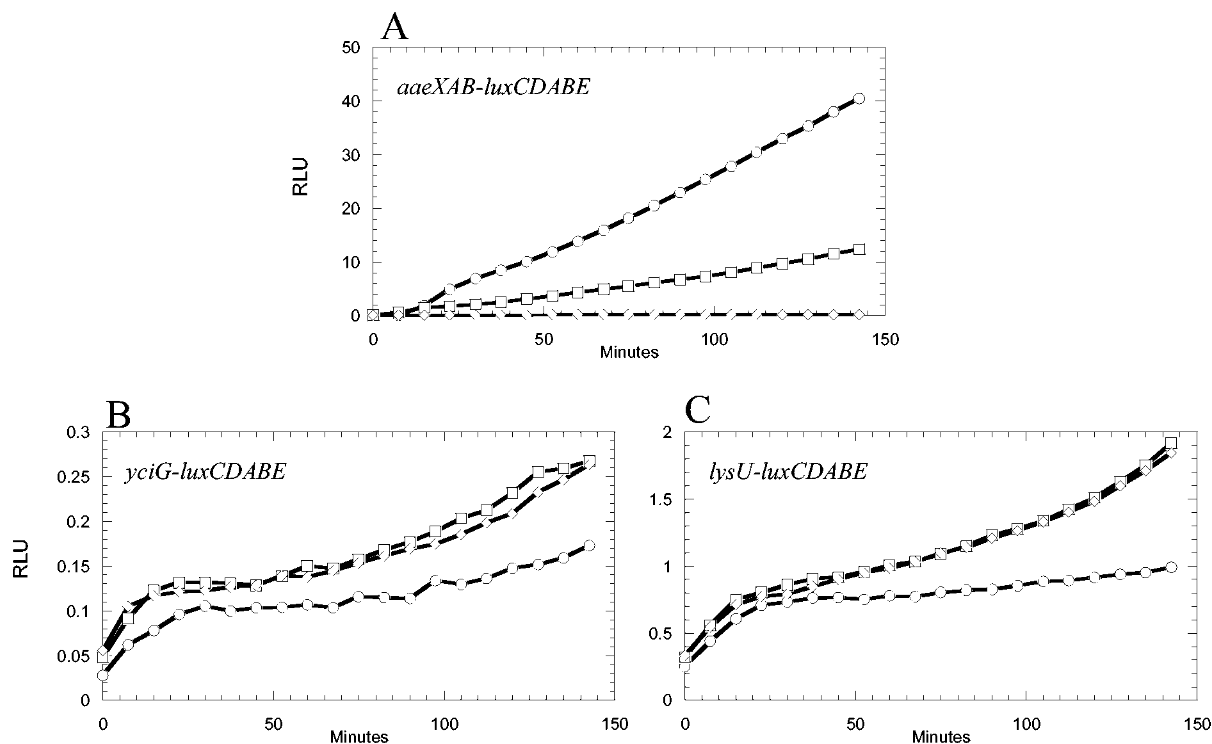

FIG. 1. Bioluminescent response of three gene fusions to salicylate. A. *E. coli* strain DPD1675 carrying plasmid pDEW655 with a *yhcRQP-luxCDABE* gene fusion. B. *E. coli* strain DPD1675 carrying plasmid pDEW215 with a *yciG-luxCDABE* gene fusion. C. *E. coli* strain DPD1675 carrying plasmid pDEW558 with a *lysU-luxCDABE* gene fusion. Diamonds, no addition. Squares, 0.6 mM sodium salicylate added at zero time. Circles, 5 mM sodium salicylate added at zero time. RLU, relative light units.

not shown). Accordingly, the requirement for the carboxylate moiety was demonstrated by the lack of response to methylparaben, the methyl ester of pHBA. Furthermore, the requirement for an aromatic ring was demonstrated by the lack of response to nonaromatic carboxylic acids, acetate and propionate. Thus, the response of this regulatory system is specific for certain aromatic carboxylic acids.

Internal acidification is not the signal that activates YhcS. The characterized activators of YhcS are weak acids. Hence, the condition inducing activation of YhcS could potentially be either cytoplasmic acidification (36) or the presence of the conjugate anion. The fact that the nonaromatic weak acids propionate and acetate, which cause cytoplasmic acidification, did not induce expression of the *yhcRQP-luxCDABE* gene fusion implies that cytoplasmic acidification is not the inducing signal. This conclusion was confirmed by experiments comparing upregulation of *yhcRQP-luxCDABE* mediated by YhcS to that of genes, such as *yciG* and *lysU*, controlled by well-known acidification-responsive regulatory circuits (41, 48). Three *E. coli* strains, each the identical host strain carrying a plasmid-borne *yhcRQP luxCDABE*, *yciG-luxCDABE*, or *lysU-luxCDABE* gene fusion, were tested. Figure 1 shows the results for salicylate addition, and Fig. 2 shows the results for acetate addition. Addition of 0.6 or 5 mM sodium salicylate upregulated expression of *yhcRQP-luxCD ABE*, but did not increase expression of the other two acid-responsive gene fusions. Conversely, treatment of *E. coli* with 80 or 160 mM acetate did not activate expression of *yhcRQP-luxCDABE*, while the expression of *yciG-luxCDABE* was increased by addition of either 80 or 160 mM acetate and expression of *lysU-luxCDABE* was elevated in the presence of 160 mM acetate. Thus, we conclude that YhcS activation of *yhcRQP* expression is not a response to acidification but rather is a response to the presence of the aromatic molecules.

Genetic evidence of YhcP efflux function. The induction of *yhcRQP* expression by aromatic carboxylic acids suggested that this class of molecules might be substrates of the YhcP efflux pump. In order to find a phenotype associated with loss of efflux function, *E. coli* strain DPD2444, containing a mutation in the *yhcP* gene, was compared with the otherwise isogenic parental strain, MG1655, for sensitivity to pHBA. The pHBA MIC for MG1655 was 100 mM, but that for the *yhcP* mutant was only 12.5 mM (Table 3). Thus, the *yhcP* mutation conferred eightfold hypersensitivity to pHBA. These results, when interpreted in the light of the efflux function predicted by informatic analysis (15), provide strong evidence that *E. coli yhcP* encodes an efflux pump for which pHBA is a substrate. Hence, the absence of this efflux pump results in increased intracellular concentrations of pHBA, which in turn is manifested as the hypersensitive phenotype.

Narrow substrate specificity of the YhcP efflux transporter. This genetic test was used to further define substrates of this efflux system. That is, likely substrates of the *yhcP* efflux pump are those compounds for which the *yhcP* mutant strain is hypersensitive compared with an otherwise isogenic control strain. *E. coli* strains DPD2444 (*yhcP*) and MG1655 ($yhcP^+$) were tested for inhibition by 240 chemicals at Biolog Inc. (Hayward, Calif.), using Phenotype MicroArrays 1 to 20, as recently described (5). These chemicals included aromatic and

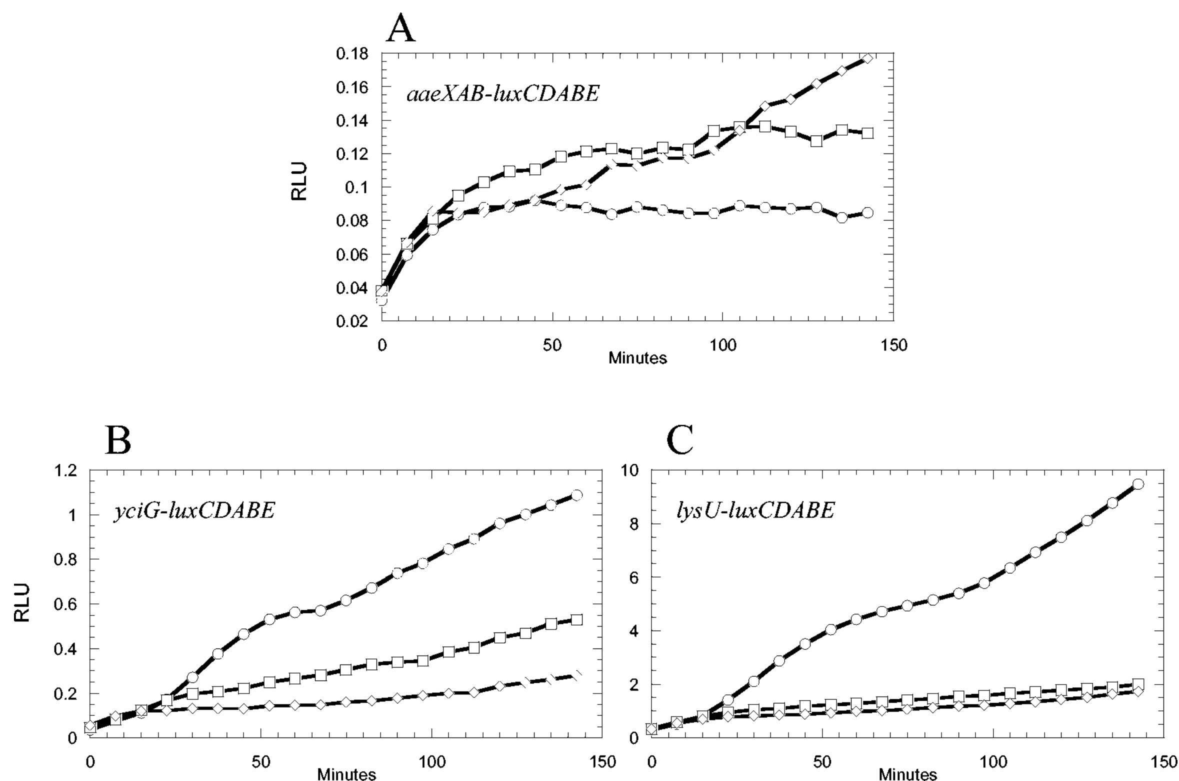

FIG. 2. Bioluminescent response of three gene fusions to acetate. A. *E. coli* strain DPD1675 carrying plasmid pDEW655 with a *yhcRQP-luxCDABE* gene fusion. B. *E. coli* strain DPD1675 carrying plasmid pDEW215 with a *yciG-luxCDABE* gene fusion. C. *E. coli* strain DPD1675 carrying plasmid pDEW558 with a *lysU-luxCDABE* gene fusion. Diamonds, no addition. Squares, 80 mM sodium acetate added at zero time. Circles, 160 mM sodium acetate added at zero time. RLU, relative light units.

heterocyclic molecules, such as acriflavin, dichloro-8-hydroxyquinoline, 9-aminoacridine, fusaric acid, salicylic acid, phenylethanol, *o*-cresol, *m*-cresol, *p*-cresol, pentachlorophenol, coumarin, DL-3-phenyl lactic acid, and cinnamic acid. Also included were numerous antibiotics and other antimicrobial compounds, including several weak acids and other compounds that would disrupt proton flux. Of all of these compounds, cinnamic acid was the only one to which strain DPD2444 was hypersensitive compared with MG1655. The score of −83 for cinnamic acid is consistent with a slight, but reproducible difference in growth inhibition between the two strains. The lack of difference between these two strains for each of the other 239 compounds tested indicates that the YhcP efflux pump has a high degree of specificity for certain aromatic carboxylic acids.

Growth inhibition of DPD2444 (*yhcP*) and *E. coli* MG1655 (*yhcP*$^+$) by additional compounds was tested (Table 3). Reliable differences between the two strains were defined as the condition with both a twofold or greater difference in MIC between the two strains and a rank (see Materials and Methods) at least 2. A slight difference in growth inhibition of the two strains by cinnamic acid was observed in this test, confirming the results from the Phenotype MicroArrays, but this difference was far less than that obtained by pHBA treatment. Accordingly, the only compounds defined as substrates of the YhcP efflux pump by this genetic test are pHBA, 6-hydroxy-2-naphthoic acid, and 2-hydroxycinnamate. Thus, this efflux system has a very high degree of specificity to certain hydroxylated, aromatic carboxylic acids.

Required components of the *yhcRQP* operon for efflux function. The nearly complete lack of induction of *yhcRQP* operon in the *yhcS* regulatory mutant (Table 2) suggests that such a mutant may be less effective at pHBA efflux. Indeed, duplicate experiments with Vogel-Bonner agar medium showed that the filter paper disks each containing 80 μmol of pHBA produced a zone of inhibition of 20.2 mm in diameter in a *yhcS* mutant (DPD2433), in comparison with 9.5 mm in the wild-type strain MG1655.

Assays of zone of growth inhibition were carried out under the same conditions with DPD2433 carrying derivatives of pTrcHis2TOPO in which various genes of the *yhcRQP* operon were expressed from the *trc* promoter. We found that the expression of the entire *yhcRQP* operon (pDEW668) or *yhcQP* genes (pDEW673) produced pHBA resistance, with inhibition zones of 9.8 and 9.5 mm, whereas expression of *yhcRQ* alone (pDEW675) produced only marginal resistance (17.0 mm) in comparison with cells containing the control plasmid, pTrcHis2TOPO/lacZ (23.5 mm). Similarly, expression of *yhcP* alone in the vector pKK223-3 did not produce any resistance (18.8 mm) in comparison with DPD2433 containing the vector alone (19.2 mm). Accordingly, expression of *yhcP* and *yhcQ* was both necessary and sufficient to fully reverse the pHBA sensitivity conferred by the *yhcS* mutation. Thus, we conclude that the products of both *yhcP* and *yhcQ* are required for

TABLE 3. Chemical sensitivities of *E. coli* strains with and without the YhcP efflux pump[a]

Chemical	MIC[b] for:		Fold difference	Rank difference
	MG1655 (*yhcP*$^+$)	DPD2444 (*yhcP*)		
pHBA	100	12.5	8	4
6-Hydroxy-2-naphthoic acid	20	2.5	8	3
2-Hydroxycinnamate	20	10	2	2
1,5-Dihydroxynaphthalene	0.5	0.25	2	1
1,6-Dihydroxynaphthalene	0.5	0.25	2	1
2,7-Dihydroxynaphthalene	0.62	0.31	2	1
2-Naphthoic acid	20	10	2	1
Cinnamic acid	20	10	2	1
1,4-Naphthalenedicarboxyylic acid	10	10	1	0
1-Hydroxy-2-naphthoic acid	0.62	0.62	1	0
1-Naphthoic acid	2.5	2.5	1	0
2,3-Dihydroxybenzoic acid	10	10	1	0
2,3-Naphthalenedicarboxylic acid	80	80	1	0
2,6-Dimethoxyphenol	5	5	1	0
3,4-Dihydroxycinnamate	0.62	0.62	1	0
3,5-Dimethoxy-4-hydroxycinnamate	10	10	1	0
3-Hydroxy-2-naphthoic acid	1.2	1.2	1	0
Benzoate	25	25	1	0
2-Biphenylcarboxlic acid	2.5	2.5	1	0
Dimethyl sulfoxide	20	20	1	0
Methyl paraben	3.8	3.8	1	0
Salicylate	100	100	1	0

[a] Cultures were grown in Vogel-Bonner minimal medium with glucose as a carbon source and tetrazolium violet to aid visualization of growth.

[b] Results for dimethyl sulfoxide are in percent, all other results are in millimolar.

function of this aromatic carboxylic acid efflux system. These results do not indicate a requirement for *yhcR* in this efflux system. However, since low-level *yhcR* expression in the *yhcS* strain is possible, such a role cannot be ruled out entirely. Several attempts to construct a nonpolar deletion of *yhcR* to further address its function were unsuccessful for unknown reasons.

Genetic evidence that that the *tolC*-encoded outer membrane factor is not required for efflux function of *yhcQP*. Numerous efflux systems in *E. coli* and other gram-negative bacteria are comprised of an inner membrane pump protein, a periplasmic MFP, and an outer membrane OMF protein (35). In analogy, with consideration that YhcQ is an MFP, it may be expected that the YhcQP efflux system for aromatic carboxylic acids would function with an OMF protein. In *E. coli*, *tolC*, encoding a OMF protein that works with several different efflux systems (30), is such a candidate. To address the role of TolC, genetic experiments using otherwise isogenic *E. coli* strains MG1655, DPD1818 (*tolC*), DPD4233 (*yhcS*), and DPD2435 (*tolC yhcS*) were conducted. These four strains were each transformed with control plasmid pTrcHis2TOPO/lacZ or with pDEW668, which expresses the *yhcRQP* operon. Examination of the strains carrying the control plasmid showed that the mutant strains had increased pHBA sensitivity compared with MG1655 (MIC, 100 mM); the pHBA MIC for *yhcS* mutant was 18 mM, that for the *tolC* mutant was 56 mM, and that for the strain carrying both the *tolC and yhcS* mutations was 13 mM. The slight pHBA hypersensitivity conferred by the *tolC* mutation suggests the presence of one or more pHBA efflux pumps in *E. coli* that utilize the TolC channel. However, if the YhcQP efflux system required TolC for function, it is expected that the pHBA MIC for the *tolC* mutant strain would have been at least as low as that for the *yhcS* mutant strain. Furthermore, the observation that the MIC for the *tolC yhcS* double mutant was lower than those for strains carrying either the *tolC* or *yhcS* mutation is consistent with independent function of TolC and the YhcQP efflux system. Moreover, the plasmid expressing *yhcRQP* conferred full pHBA resistance (MIC, 100 mM) in all host strains, including the *tolC* mutant hosts. Thus, these results indicate that the YhcP efflux pump does not require exclusive use of TolC. Overall, these results can be interpreted as being consistent with the presence of at least two pHBA efflux systems in *E. coli*, one that uses TolC and the YhcQP system, which does not.

DISCUSSION

The results of this study, considered in light of the phylogenetic analysis of Harley and Saier (15), provide strong evidence for the function of YhcP in efflux of aromatic carboxylic acids. Thus, this is the first experimental demonstration of efflux function for a member of the PET protein family. Additionally, we have demonstrated the requirement of YhcQ, an MFP, for YhcP efflux function. Furthermore, the regulation of expression of the *yhcRQP* operon by YhcS was demonstrated. However, no functional role of *yhcR*, encoding a small protein, was found in these studies. Nonetheless, the association of *yhcR* with *yhcQ* and *yhcP* in the *E. coli* genome hints that it may be important for function of these proteins in some manner.

In light of the demonstrated function in aromatic carboxylic acid efflux or regulation of expression of the efflux genes, we propose renaming these genes with a mnemonic for aromatic carboxylic acid efflux as follows: *aaeB* (*ychP*), *aaeA* (*yhcQ*), *aaeX* (*yhcR*), and *aaeR* (*yhcS*). Other researchers have suggested an alternative designation for *yhcS* (b3243) of *qseA* in light of twofold upregulation in response to quorum sensing (44). However, the role in regulation of the *aeeXAB* efflux operon is clearly biologically relevant, while there are complications in interpretation of cell-to-cell signaling in *E. coli* (1), and thus the designation *aaeR* is appropriate. Nevertheless, a role of AaeR in quorum sensing cannot be ruled out from our data.

The AaeAB efflux system is distinct from many efflux pumps in *E. coli* that use the TolC channel-tunnel, as we found no evidence that TolC was required for AaeAB function. At present, it is not known whether one of the other OMF family members present in the *E. coli* genome, i.e., CusC, YohG, or YjcP, works with the AaeAB efflux system. Alternatively, this efflux system may use another type of outer membrane protein for efflux or may not require an outer membrane component. Interestingly, AaeA is predicted to have an alpha-helical barrel similar to that present in TolC; however, the significance of the predicted structure is not known (38).

Likewise, the significance of *aaeX* is not known. However, it is interesting to consider that in the CusCFBA copper and silver efflux system, the 10-kDa CusF protein is a periplasmic copper binding protein that is required for maximal efficiency of copper efflux (13). Thus, it is possible that AaeX may play a

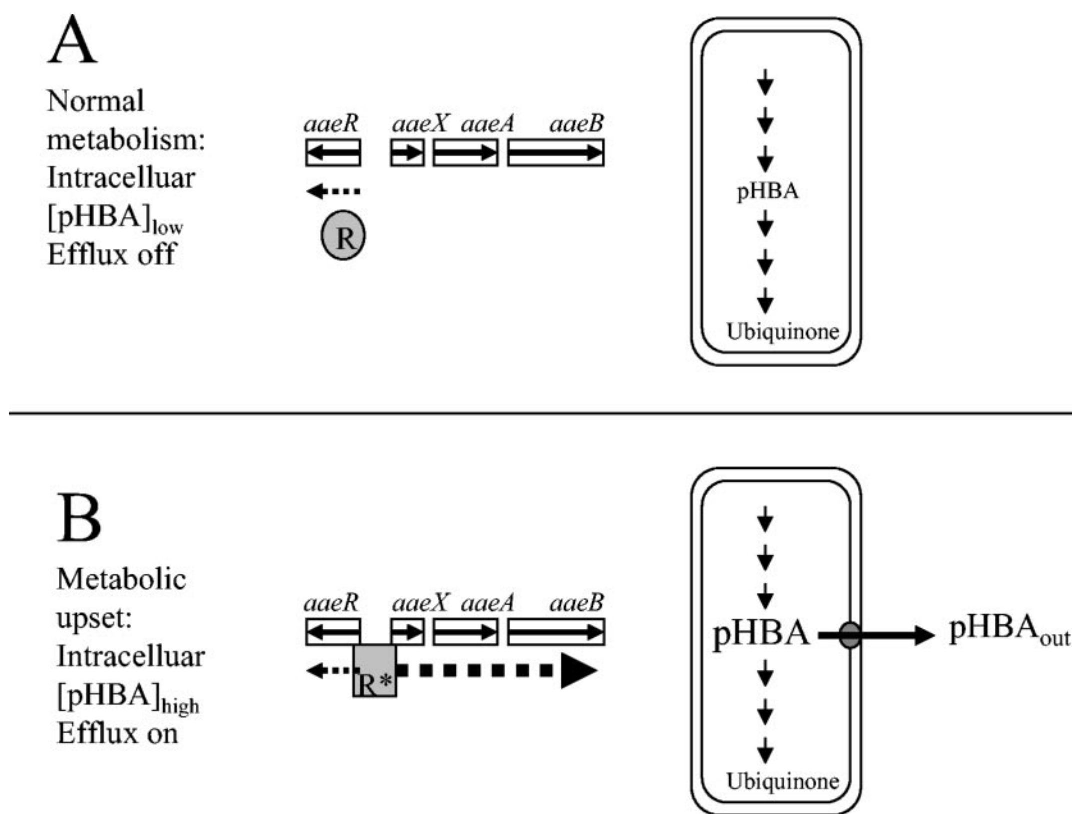

FIG. 3. Metabolic relief valve model for AaeAB efflux pump expression. A. In normal *E. coli* cellular metabolism, pHBA is an intermediate in ubiquinone biosynthesis, and its intracellular concentration is likely very low. In this situation, the regulatory gene *aaeR* is expressed, but the positive regulator is in an inactive form as depicted by the circle. Accordingly, expression of the genes encoding the efflux pump, *aaeA* and *aaeB*, which requires the active form of the regulatory protein for expression, is very low. B. In a situation of a metabolic upset when the intracellular concentration of pHBA is abnormally high, the AaeR regulator binds pHBA and is converted to the active form as depicted by the square, thus activating transcription of the efflux pump genes. Expression of the efflux pump proteins thus results in removal of excess pHBA and hence in restoration of cellular homeostasis.

similar role in the efflux mediated by AaeAB but was not detected in our experiments.

The substrate specificity of the AaeAB efflux system is very narrow, being limited to hydroxylated aromatic carboxylic acids. This is in distinct contrast to multidrug efflux systems, such as AcrAB-TolC. The substrate specificity for inducers of *aeeXAB* expression and thus presumably for binding to the AeeR regulator is somewhat broader in that both nonhydroxylated and hydroxylated aromatic carboxylic acids are active. Nonetheless, pHBA is the best inducer and the best substrate among the compounds studied. Of course, pHBA is an intermediate of ubiquinone biosynthesis and thus would normally be expected to be present at very low concentrations in *E. coli* cells. It is also notable that the basal, "off," level of *aaeXAB* expression is very low. The bioluminescence of the *aaeXAB-luxCDABE* reporter in the absence of inducing molecules is very low. Also, the steady-state level of *aaeXAB* mRNA in minimal or rich medium, estimated from hybridization assays normalized with cellular DNA, is at or slightly above detection limits (52). In contrast, the steady-state expression level of the *aaeR* regulatory gene as quantitated by an *aaeR-luxCDABE* gene fusion (Van Dyk, unpublished data) or as measured in the normalized hybridization assay (R. A. LaRossa, personal communication) is substantial. Considering all these observations, we suggest a role of the AaeAB efflux system as a "metabolic relief valve." Accordingly, these proteins are not normally expressed to any significant level, but if a metabolic upset occurs such that pHBA accumulates to high levels in the cell, the expression of the efflux system is activated (Fig. 3). Thus, this highly regulated efflux system may normally function as a response to an intracellular stress condition rather than to an externally imposed stress.

The modes of pHBA toxicity are not well defined. However, changes in fatty acid composition in *Pseudomonas putida* upon pHBA treatment point to a membrane site of action (32). Furthermore, in general, imbalances of normal cellular metabolites lead to physiological upsets (20). Accordingly, other efflux transporters with substrates that are cellular metabolites may likewise have a metabolic relief valve function. Such a role has been proposed for amino acid efflux systems (7). Thus, the *Corynebacterium glutamicum* LysE lysine effluxer (3, 6), ThrE threonine and serine effluxer (40), and BrnFE isoleucine and leucine effluxer (17) and the *E. coli* RhtA, RhtB, and RhtC threonine effluxers (19, 21, 53) and ArgO arginine effluxer (26) may serve a role in pumping excess amino acids out to the cell. Similarly, the *E. coli* EamA (YdeD) (8) and EamB (YfiK) (11) transporters, which efflux cysteine pathway metabolites, may also play a role in ridding the cell of excess metabolites that

would otherwise disrupt metabolism. Furthermore, even efflux systems with broad substrate specificity, such as AcrAB-TolC, have been suggested to play a role in general toxic metabolite efflux (16). Thus, it is becoming increasingly evident that efflux transport plays an important role in controlling levels of normal metabolites in bacterial cells. Furthermore, although these are microbial examples, it seems reasonable to consider that such a function would also be found in higher organisms.

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