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Role of the AheABC Efflux Pump in *Aeromonas hydrophila* Intrinsic Multidrug Resistance[▽]

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Gene inactivation and complementation experiments showed that the tripartite AheABC efflux pump of *Aeromonas hydrophila* extruded at least 13 substrates, including nine antibiotics. The use of phenylalanine-arginine-β-naphthylamide (PAβN) revealed an additional system(s) contributing to intrinsic resistance. This is the first analysis of the role of multidrug efflux systems in *Aeromonas* spp.

Aeromonas hydrophila is a gram-negative bacillus living in a variety of aquatic environments (1). This organism is occasionally responsible for human infections following contact with contaminated water or derived products. Clinical manifestations include gastroenteritis, skin and soft tissue infections, and a series of clinical syndromes that appear in immunocompromised patients (11). Among *Aeromonas* spp., multidrug resistance (MDR) phenotypes were observed (7, 8, 9), some of which were reversed by the efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAβN) (7, 14). To date, no functional data are available for MDR efflux pumps in *Aeromonas* species. However, these systems, especially those belonging to the resistance-nodulation-cell division (RND) family, are known to play a major role in the antimicrobial resistance of various environmental gram-negative bacilli that behave as opportunistic pathogens (18).

The 4.7-Mb genome of *A. hydrophila* subsp. *hydrophila* ATCC 7966^T has recently been sequenced (21). Ten RND systems have been detected, including seven belonging to the hydrophobe/amphiphile efflux-1 family (20, 21). Among them, AHA2910, renamed AheB (*Aeromonas hydrophila* efflux B), is the pump most closely related to that of the major AcrB system of *Escherichia coli*. The *aheA* (1,194 bp) and *aheC* (1,434 bp) genes are located immediately upstream and downstream of *aheB* (3,150 bp), respectively. *aheA*, *aheB*, and *aheC* are in the same orientation and are organized in an operon-like structure (Fig. 1). Their deduced protein sequences share the highest identities with the membrane fusion protein AcrA of *Photorhabdus luminescens* (54%), the RND efflux pump AcrB of *E. coli* (69%), and the outer-membrane protein TtgC of *Pseudomonas entomophila* (58%), respectively. Another open reading frame (642 bp), called *aheR*, situated 143 bp upstream and in the opposite orientation of *aheABC*, exhibits sequence identity with the transcriptional repressors of the TetR family, such as AcrR of *E. coli* (37.6%) (Fig. 1).

The aims of the present study were to investigate the role of the tripartite RND efflux pump AheABC in the intrinsic resistance of *A. hydrophila* ATCC 7966^T and to delineate its substrate specificity by inactivation of the *aheB* gene and subsequent AheABC complementation experiments.

At first, the transcription of the *aheB* gene in *A. hydrophila* ATCC 7966^T (collection of Institut Pasteur, France) was assessed by reverse transcription (RT)-PCR amplification. RNA extracts (2 ng) were prepared from logarithmic and late-growth-phase cultures in Mueller-Hinton (MH) broth, with an SV Total RNA Isolation kit that includes a DNase treatment (Promega, France). The oligonucleotides used, AheB1F1 and MotifD1 (Table 1), targeted an internal fragment of *aheB* (Fig. 1). RT-PCR experiments were carried out with and without (as control) reverse transcriptase in a single RT-PCR system tube, using an AccessQuick RT-PCR kit and RT-PCR conditions as recommended by the manufacturer (i.e., one RT step of 45°C for 45 min and then 40 cycles of 94°C for 30 s, 60°C for 1 min, and 68°C for 1 min) (Promega). An RT-PCR fragment with the expected size of 947 bp was obtained for cells grown at both growth phases, demonstrating that *aheB* was constantly transcribed in *A. hydrophila* ATCC 7966^T (data not shown). Further analysis by Northern blotting and/or quantitative RT-PCR would be necessary to evaluate expression variations at different growth phases, as previously shown for *mexB* in *Pseudomonas aeruginosa* (6).

Since *aheB* was expressed in cells grown in standard laboratory medium, in contrast to other pumps (13, 16), its inactivation could be performed to examine the resulting hypersensitive phenotype. Thus, two PCR products with restriction sites at their ends were amplified from the *aheB* gene, i.e., the amplicon A, using the primer pairs motifAF_KpnI and motifAR_SalI and the amplicon D, using the primer pairs motifDF_BamHI and motifDR_SphI (Table 1). The digested A and D fragments were ligated with the *aph3′* gene (conferring kanamycin resistance [Kan^r]) amplified by the primer pair kan1_SalI and kan2_BamHI from the pTnModOKan plasmid (Table 1 and Fig. 1) (4). The resulting AKD fragment was inserted into the KpnI and SphI double-digested plasmid pEX19Gm, generating the pEX-AKD plasmid. This vector was introduced by electroporation into the *E. coli* strain SM10 (Belgian Coordinated Collections of Microorganisms, Belgium), which is able to *trans* mobilize plasmids containing an *oriT* transfer origin, such as pEX19Gm (10). Conjugation experiments between the recombinant *E. coli* SM10 as the donor strain and *A. hydrophila* (intrinsically ampicillin resistant) as the recipient strain were

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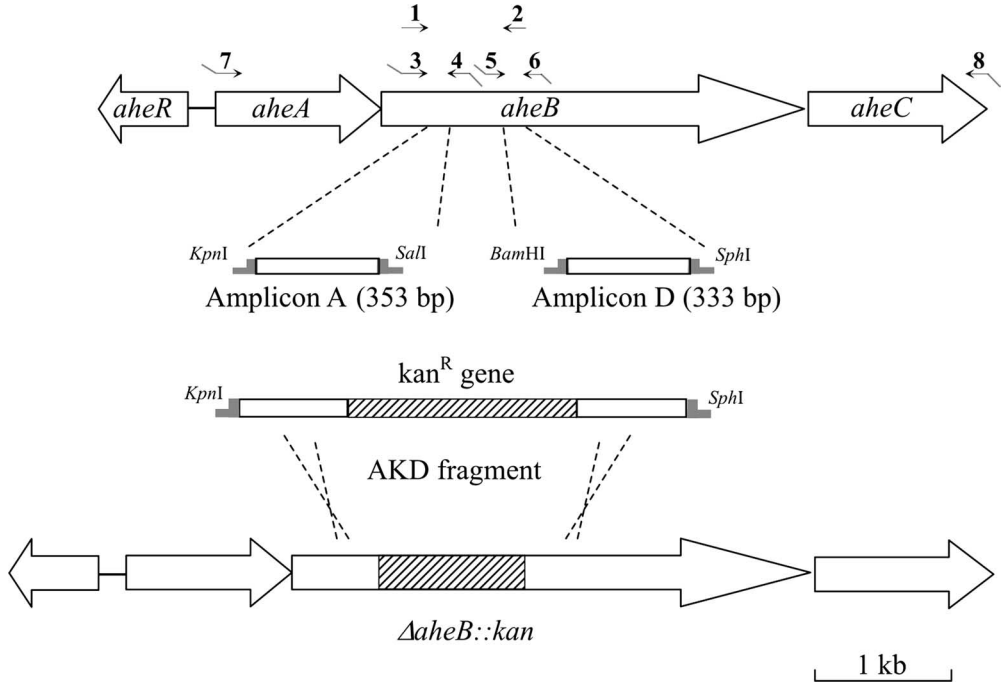

FIG. 1. Schematic representation of the *aheRABC* gene cluster and *aheB* inactivation strategy. Open arrows show the position and orientation of the *aheR*, *aheA*, *aheB* and *aheC* genes. At the top of the figure, the horizontal thin arrows indicate the position and orientation of primers used for amplification experiments and are numbered as follows: 1, aheB1F1; 2, MotifD1; 3, MotifAF_KpnI; 4, MotifAR_SalI; 5, MotifDF_BamHI; 6, MotifDR_SphI; 7, yAheAdbt and 8, yAheCfnR. Oligonucleotides with a 5′-end modification (i.e., introduction of restriction site or DNA yeast sequences) are represented by horizontal thin black arrows with a tail. The amplicons A and D (white boxes) possess cohesive ends (in gray) when digested by KpnI and SalI (amplicon A) and by BamHI and SphI (amplicon D) restriction enzymes. After ligation with the kanamycin resistance gene (kan^R, as a hatched box), the AKD fragment was obtained. By double crossover between the chromosomal *aheB* gene and the homologous sequences situated on pEX-AKD plasmid, a Δ*aheB*::*kan* mutant was constructed.

performed by using a filter-mating procedure (3). *A. hydrophila* transconjugants were obtained on selective MH agar medium supplemented with kanamycin (50 mg/liter) plus ampicillin (100 mg/liter) at a transfer frequency of ca. 10^{-5} per donor cell. However, it was found that the plasmids PEX19Gm and pEX-AKD were able to replicate in *A. hydrophila*, in contrast to those in *P. aeruginosa* (10) (data not shown). Then, *aheB* was inactivated by using the *sacB*-based strategy: the *sacB* gene located on pEX19Gm and pEX-AKD codes for a levansucrase that yields toxic metabolites at high sucrose concentrations

TABLE 1. Primers used for PCR amplification

Primer name	Primer orientation (5′ → 3′)[a]	Nucleotide position
RT-PCR amplification		
AheB1F1	GGTGCAGGTGCAGAACAAGCTGCAG	2308–2332[b]
MotifD1	TCAGGCGCTCCACGTTCTC	3236–3254[b]
aheB inactivation		
MotifAF_KpnI	CACACAGGTACCAGGTGCAGGTGCAGAACAAGCTG	2307–2329[b]
MotifAR_SalI	CACACAGTCGACCCCAGCTTGCCAAAGGCCACCTG	2633–2655[b]
MotifDF_BamHI	CACACAGGATCCCTTCCGCGCCACGCTGATCCCG	3079–3100[b]
MotifDR_SphI	CACACAGCATGCCGAGAACTGGCGATAGATGGC	3389–3409[b]
Kan1_SalI	CACACAGTCGACGCCGAGCTTTGTCATCACC	2735–2754[c]
Kan2_BamHI	CACACAGGATCCTTAGAAAAACTCATCGAGCAT	1096–1116[c]
aheABC cloning in yeast cells		
yAheAdbt	AGCCCGGGGGATCCACTAGTTCTAGAA**GGAGG**TTCTCC**ATG**CATAAACATATTCTCGC[d]	786–803[b]
yAheCfnR	ACTCACTATAGGGCGAATTGGAATTCCTACTGCTGACGCTCGCCCTTG	6533–6554[b]

[a] The first 5′ base is marked by a double underline. The positions of the enzyme restriction sites in the primers are underlined (KpnI, MotifAF_KpnI; SalI, MotifAR_SalI and Kan1_SalI; BamHI, Kan2_BamHI and MotifDF_BamHI; SphI, MotifDR_SphI; XbaI, yAheAdbt; EcoRI, AheAdbt).
[b] DNA sequence deposited under GenBank accession number EF613320.
[c] DNA sequence deposited under GenBank accession number AF061921.
[d] The positions of the ribosome binding site and the ATG initiation codon of the *aheABC* operon are indicated in bold.

TABLE 2. Susceptibility to antibiotics and nonantibiotic compounds

Compound	MICs (mg/liter) for strains with native or recombinant plasmids[a]						
	A. hydrophila ATCC 7966^T (+ PAβN)[b]			*E. coli* TG1			
	Wild-type strain + pEX19Gm	Δ*aheB*::*kan* strain + pEX19Gm	Δ*aheB*::*kan* strain + pEXAheABC	Wild-type strain + pEX19Gm	Δ*acrAB* strain + pEX19Gm	Δ*acrAB* strain + pUCAcrAB	Δ*acrAB* strain + pEXAheABC
Cefuroxime	1 (1)	0.25 (0.25)	4 (4)	4	0.5	8	2
Cefoperazone	0.25 (0.25)	0.06 (0.06)	2 (2)	0.25	0.03	8	0.06
Erythromycin	16 (2)	8 (1)	64 (2)	64	1	128	32
Lincomycin	128 (128)	64 (64)	256 (256)	1,024	64	>1,024	512
Pristinamycin	32 (2)	8 (1)	128 (4)	64	4	64	64
Minocycline	1 (0.5)	0.4 (0.25)	2 (1)	1	0.12	2	1
Trimethoprim	16 (8)	8 (4)	32 (16)	0.2	0.05	0.05	0.05
Fusidic acid	128 (2)	64 (1)	256 (8)	512	2	512	128
Rifampin	2 (0.06)	1 (0.03)	4 (0.12)	16	8	16	8
Ethidium bromide	128 (128)	64 (64)	512 (512)	512	2	512	64
Acridine orange	512 (256)	256 (128)	1,024 (256)	>1,024	128	>1,024	256
Benzalkonium chloride	64 (64)	32 (32)	64 (64)	128	4	128	8
Tributyltin	32 (2)	16 (2)	256 (4)	>1,024	4	>1,024	16

[a] MICs were determined from experiments performed at least in triplicate, and the mode values were retained.
[b] MICs indicated in parentheses were determined in the presence of 64 mg/liter of PAβN (i.e., to a subinhibitory concentration corresponding to one-fourth the MIC for the *A. hydrophila* strain).

(10). Accordingly, after growth on a selective medium containing a 10% lethal concentration of sucrose, one of the survivors, the Δ*aheB*::*kan* mutant, was selected. PCR amplification experiments, followed by sequencing, revealed that the kanamycin cassette was correctly inserted in chromosomal DNA at amino acid position 220 of AheB, leading to the deletion of amino acids 221 to 361 (data not shown).

To outline the substrate specificity of the AheABC efflux pump, complementation experiments were necessary. Despite several independent experiments, cloning a PCR product of the entire *aheABC* operon directly into pEX19Gm remained unsuccessful. Thus, we performed a previous cloning step with *Saccharomyces cerevisiae*. First, using a Phusion DNA polymerase (Finnzymes, Finland), the operon was amplified with the primer pair yAheAdbt and yAheCfnR (Table 1) (Fig. 1). The resulting fragment of 5,769 bp contained XbaI and EcoRI recognition restriction sites and an usual prokaryotic ribosome binding site (GGAGG) 6 bp upstream from the ATG initiation codon (Table 1). This fragment was mixed with the linearized yeast-bacteria shuttle vector pGB6 (an unpublished vector derived from pRS316, including *URA3*, encoding a uracil biosynthesis enzyme, and $bla_{\text{TEM-1}}$, encoding ampicillin resistance). Cells of *S. cerevisiae* BY4742 (*ura3Δ0*; EUROpean *S. cerevisiae* ARchive for Functional collection, Germany) were transformed with the *aheABC* fragment and the pGB6 plasmid according to a one-step protocol based on an incubation of 30 min at 45°C in a medium containing thio compounds and alkali ions (2). The presence of the same 20-bp sequence at the ends of the amplicon (Table 1, primers yAheAdbt and yAheCfnR) and at the ends of the linearized vector allowed an efficient homologous recombination in yeast and led to the formation of the replicative circular plasmid pGB-AheABC. Yeast plasmids were extracted from the uracil-positive clones by a previously described procedure (19), and one of them was transferred by electroporation to *E. coli* SM10. After double digestion with XbaI and EcoRI, the insert of pGB-AheABC was introduced into pEX19Gm in the same orientation as the resident *lacZ* promoter for optimal expression, generating the pEX-AheABC plasmid. After verification by sequencing, this recombinant plasmid, which conferred gentamicin resistance due to the presence of an *aacC1* determinant located on pEX19Gm was transferred by conjugation to the Δ*aheB*::*kan* mutant. Thus, *Aeromonas* transconjugants were selected and cultivated on an MH medium containing ampicillin (100 mg/liter) and gentamicin (10 mg/liter).

Aeromonas strains with native or recombinant plasmids were compared first by the agar diffusion method, using all 53 available antibiotic disks (amikacin, amoxicillin, amoxicillin-clavulanic acid, ampicillin, aztreonam, cefepime, cefoperazone, cefotaxime, cefotetan, cefoxitin, cefpirome, cefsulodin, ceftazidime, cefuroxime, cephalothin, chloramphenicol, ciprofloxacin, clindamycin, colistin, doxycycline, erythromycin, fosfomycin, fusidic acid, gentamicin, imipenem, levofloxacin, lincomycin, minocycline, moxalactam, nalidixic acid, netilmicin, nitrofurantoin, nitroxolin, norfloxacin, ofloxacin, oxacillin, penicillin, pipemidic acid, piperacillin, piperacillin-tazobactam, pristinamycin, rifampin, sparfloxacin, spectinomycin, spiramycin, streptomycin, sulfamethoxazole, tetracycline, ticarcillin, ticarcillin-clavulanic acid, tobramycin, trimethoprim, and trimethoprim-sulfamethoxazole; Bio-Rad, France), corresponding to drugs currently in use for the treatment of human infections (15). Then, MICs were determined by an agar dilution method in MH medium, with a final inoculum of about 10^4 CFU per spot (15), for the nine compounds that showed variations (cefuroxime, cefoperazone, erythromycin, lincomycin, pristinamycin, minocycline, trimethoprim, fusidic acid, and rifampin). The MICs of 10 nonantibiotic agents were also determined. Four of them gave varied results (ethidium bromide, acridine orange, benzalkonium chloride, and tributyltin), while six gave no changes (sodium dodecyl sulfate, rhodamine 6G, crystal violet, sodium deoxycholate, tetraphenylphosphonium chloride, and acriflavine) (Table 2). The differences in MICs between the wild-type *A. hydrophila* strain and its hypersensitive mutant did not exceed fourfold, suggesting that AheB expres-

sion occurred at a moderate level in the wild-type strain. Consequently, AheABC might not be as critical in the species-specific resistance of *A. hydrophila* as AcrAB is in *E. coli* (23). Alternatively, these low MIC differences might be ascribed to the overproduction of other efflux pumps or a regulatory system(s) in *A. hydrophila* to compensate the nonfunctional AheB protein, as described for AcrB mutations in *Salmonella enterica* serovar Typhimurium (5). When the multicopy pEX-AheABC plasmid was introduced into the Δ*aheB*::*kan* mutant, the same compounds were affected, but differences in MICs were often higher, reaching 33-fold for cefoperazone and 16-fold for cefuroxime, pristinamycin, and tributyltin, and thus were considered the preferential substrates of AheB (Table 2).

In order to compare the substrate profiles of AheABC and AcrAB, pEX-AheABC was introduced into *E. coli* TG1Δ*acrAB* (17). AheABC retained its substrate specificity in this heterologous host, except for rifampin and trimethoprim, which were not affected. The increases in MICs of some substrates (acridine orange and tributyltin) were lower in *E. coli* than in *A. hydrophila* strains, whereas the reverse was noted for other drugs (erythromycin, fusidic acid, and ethidium bromide). Consequently, these variations did not seem to be related to different levels of AheABC expression in both bacteria but rather resulted from differences in outer-membrane permeability, as previously suggested for MexAB-OprM in *E. coli* and *P. aeruginosa* (22). MIC variations for the two *E. coli* isogenic Δ*acrAB* and Δ*acrAB*(pUCAcrAB) strains (17) were higher than those for the Δ*acrAB* and Δ*acrAB*(pEXAheABC) strains for all AheABC substrates except pristinamycin and trimethoprim (Table 2). Thus, even if both RND pumps share sequence homologies, AheABC appears to have a narrower range of substrates than AcrAB-TolC (23). Indeed, in contrast to the latter pump, AheABC did not extrude acriflavine and quinolones, although RND family exporters are commonly involved in fluoroquinolone resistance in gram-negative bacteria (18). Similarly, AheABC did not accommodate bile salts, due maybe to the fact that the usual environment of this bacteria is not, as for *E. coli*, the human digestive tract (1). In contrast, AcrAB exported tributyltin much more efficiently than AheABC did, although this antifouling agent is a common pollutant of water sediments that can occasionally contaminate the *Aeromonas* sp. ecosystem (12).

The addition of PAβN (Sigma-Aldrich, France) (14) did not modify the MICs of some AheABC substrates (cefuroxime, cefoperazone, lincomycin, ethidium bromide, and benzalkonium chloride), whereas it strongly decreased the MICs of others, whether they were preferential substrates of this pump or not (Table 2). The considerable potentiation of some compounds by PAβN in the wild-type *A. hydrophila* strain as well as in its Δ*aheB*::*kan* mutant suggested that this inhibitor might block an additional RND efflux system(s).

In conclusion, this report is the first functional analysis of a multidrug efflux pump belonging to the RND family in *Aeromonas* spp. The AheABC system is involved in the MDR phenotype of *A. hydrophila*, albeit at low levels. However, inhibition tests using PAβN indicate that other RND-type efflux pumps also contribute to the intrinsic drug resistance of this species. Further experiments, facilitated by the methodology developed in this work, are warranted to explore the type of transport, substrate profile, and regulation mechanisms of these systems.

Nucleotide sequence accession number. The nucleotide sequence of the 6,554-bp fragment containing the *aheRABC* genes of *A. hydrophila* ATCC 7966^T has been deposited in the GenBank/EMBL sequence database under accession number EF613320.

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