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The *eefABC* Multidrug Efflux Pump Operon Is Repressed by H-NS in *Enterobacter aerogenes*

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The *Enterobacter aerogenes eefABC* locus, which encodes a tripartite efflux pump, was cloned by complementation of an *Escherichia coli tolC* mutant. *E. aerogenes* Δ*acrA* expressing EefABC became less susceptible to a wide range of antibiotics. Data from *eef*::*lacZ* fusions showed that *eefABC* was not transcribed in the various laboratory conditions tested. However, increased transcription from P*eef* was observed in an *E. coli hns* mutant. In addition, EefA was detected in *E. aerogenes* expressing a dominant negative *E. coli hns* allele.

During the last decade *Enterobacter aerogenes* has emerged as an important nosocomial pathogen, usually affecting immunocompromised patients. This gram-negative bacterium is now the third most common pathogen recovered from the respiratory tract and is often isolated in the urine and gastrointestinal tracts (31). *E. aerogenes* strains isolated from hospitalized patients generally exhibit high resistance levels to a wide variety of antibiotics, including β-lactams, quinolones, chloramphenicol, and tetracyclines (5, 9, 20). In *E. aerogenes* and other gram-negative bacteria, the decrease of outer membrane permeability and the induction of active drug efflux contribute to multidrug resistance (MDR) (6, 20, 24). The well-studied AcrAB-TolC and MexAB-OprM multidrug efflux pumps are responsible for MDR in *Escherichia coli* and *Pseudomonas aeruginosa*, respectively (18, 26). These pumps belong to the resistance-nodulation-division (RND) family (27). RND-type drug efflux pumps share a common three-component organization across the two membranes: a periplasmic linker protein (AcrA, MexA), an inner membrane transporter (AcrB, MexB), and an outer membrane channel (TolC, OprM). The recent elucidations of the crystal structures of TolC and AcrB from *E. coli* and MexA from *P. aeruginosa* gave rise to major progress in the understanding of the efflux mechanism in gram-negative bacteria (13, 16, 22).

The entire *E. aerogenes* genome has yet to be sequenced; therefore, complementation was used to clone the drug efflux systems of *E. aerogenes*. An *E. aerogenes* genomic library has previously been screened for complementation of *E. coli acrAB* or *tolC* mutants. The *E. aerogenes acrAB* and *tolC* loci had been identified, and the AcrAB-TolC pump was shown to contribute to MDR in an *E. aerogenes* clinical isolate (29). In the present study, we used the same complementation approach to clone a novel multidrug efflux system of *E. aerogenes*, which we named EefABC (for *Enterobacter* efflux).

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We found that *eefABC* expression is silent in laboratory growth conditions but induced in both an *E. coli hns* mutant and an *E. aerogenes* strain expressing a dominant negative H-NS protein from *E. coli*.

Cloning of the *E. aerogenes eefABC* operon and sequence analysis. We used a genomic library of *E. aerogenes* BW16627 in the form of a mini-Mu dI5166 lysate to complement *E. coli* EP663 *tolC* for growth on plates supplemented with 0.05% deoxycholate (DOC) (11, 29, 34). Among eight recombinant plasmids containing overlapping DNA fragments, we selected pEP770, which carries the shortest insert of about 13.5 kb. *E. coli* EP665 *acrAB tolC* was constructed by P1 transduction of EP663 (*tolC*::Tn*10*) with a phage lysate prepared on EP661 (Δ*acrAB*::Km^r). In contrast to *acrAB tolC* mutants, which are hypersusceptible to hydrophobic compounds (11, 26,34), EP665(pEP770) was able to grow on plates containing sodium dodecyl sulfate (SDS; 0.1%), DOC (0.05%), novobiocin (3 μg/ml), erythromycin (5 μg/ml), acriflavin (200 μg/ml), or ethidium bromide (10 μg/ml). This suggests that the *E. aerogenes* DNA insert in pEP770 encodes a complete tripartite efflux pump and not only a TolC homologue. Strains and plasmids used are shown in Table 1.

The nucleotide sequence of an 8-kb DNA region from pEP770 was determined and analyzed. Among the six open reading frames identified, the three adjacent, *orf3*, *orf4*, and *orf5*, were found to be homologous to efflux pump genes and were named *eefABC*. The *eef* genes are tightly linked and are probably transcribed as an operon. EefA shares 52% and 49% sequence identity with the MexA (*P. aeruginosa*) and AcrA (*E. aerogenes* and *E. coli*) periplasmic linker proteins, respectively. The N-terminal region of EefA exhibits characteristics of a signal sequence including a consensus lipoprotein-processing site (LSGC) (36). EefB shares 56% and 57% sequence identity with the MexB (*P. aeruginosa*) and AcrB (*E. aerogenes* and *E. coli*) inner membrane transporters, respectively. EefC presents 43%, 24%, and 22% sequence identity with the OprM (*P. aeruginosa*), TolC (*E. aerogenes*), and TolC (*E. coli*) outer membrane proteins, respectively. OprM has been shown to be acylated (23), and several *P. aeruginosa* OprM homologues are predicted to be lipoproteins as they all possess a characteristic lipoprotein box with a conserved cysteine residue immediately

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description[a]	Source or reference
E. coli		
BW5104	Mu-1 Δ*lac-169 creB510 hsdR514*	17
EP661	BW5104 Δ*acrAB*::Km^r	29
EP663	BW5104 *tolC*::Tn*10* Tc^r	29
EP665	BW5104 Δ*acrAB*::Km^r *tolC*::Tn*10* Tc^r	This work
S17.1 λ*pir*	*recA thi pro hsdRM*$^+$ RP4::2-Tc::Mu::Km Tn7 lysogenized with λ *pir* phage	10
PS2209	Wild type	4
PS2652	PS2209 *hns-1001* Sm^r	4
E. aerogenes		
BW16627	ATCC 15038 *rpsL* Sm^r Amp^r	17
BW16662	ATCC 15038(pREG2-1, pEG5166S) Cm^r	17
BW16665	ATCC 15038(pREG2-1, pEG5166S) Cm^r	17
EAEP295	Nal^r derivative of BW16627	29
EAEP289	Km^s derivative of the EA27 MDR clinical isolate, Ap^r Cm^r Nal^r Sm^r Tc^r	29
EAEP308	EAEP289 Δ*acrA*	29
EAEP60	EAEP295 *eefA*::*lacZ* Km^r	This work
EAEP62	EAEP289 *eefA*::*lacZ* Km^r	This work
EAEP64	EAEP308 *eefA*::*lacZ* Km^r	This work
Plasmids		
Mu dI5166	Mini-Mu for in vivo cloning, Cm^r	12
pEP770	Mu dI5166 containing *eefABC* on a 13.5-kb insert, Cm^r	This work
pBCSK$^+$	High-copy-number cloning vector, Cm^r	Stratagene
pDrive	High-copy-number cloning vector, Ap^r Km^r	Qiagen
pEP867	pDrive containing *eefABC* on a 6-kb SspI insert, Ap^r Km^r	This work
pVIK112	*pir*-dependent *oriR6K,* suicide vector for *lacZ* transcriptional fusion, Km^r	15
pEP872	pVIK112 containing the *eefABC* promoter region on a 1.2-kb PstI-SacII fragment, Km^r	This work
pGEM-T	High-copy-number PCR cloning vector, Ap^r	Promega
pEP137	pGEM-T containing the *eefABC* promoter region on a 0.6-kb insert, Ap^r	This work
pFus2	Promoterless *lacZ* cloning vector, Gm^r	1
pUC4K	Source of Km^r cassette	Amersham Biosciences
pFus2-K	Km^r Gm^s derivative of pFus2, obtained by insertion of a BamHI Km^r cassette into the BglII site of the Gm^r gene of pFus2	This work
pMM38	pFus2-K containing the *eefABC* promoter region on a 0.6-kb BamHI fragment, Km^r	This work
pSU19	pACYC184 derivative, Cm^r	2
pDIA547	pSU19 containing the *E. coli hns* gene, Cm^r	4
pLG339	Low-copy-number cloning vector, *ori-pSC101* Tc^r Km^r	33
pLGH-NSL26P	pLG339 containing an *E. coli* dominant negative *hns* allele, Km^r Tc^r	35

[a] Ap^r, Cm^r, Gm^r, Km^r, Sm^r, and Tc^r, resistance to ampicillin, chloramphenicol, gentamicin, kanamycin, streptomycin, and tetracycline, respectively.

downstream of an N-terminal signal sequence (28). EefC contains such a lipoprotein box (*C*VSL); thus, it may be acylated.

EefABC is an MDR efflux pump in *E. aerogenes*. When *eefABC* was cloned into the pDrive vector downstream from the *lac* promoter, the resulting plasmid conferred substantial restoration of antibiotic resistance to EAEP308, although not to the levels of the AcrA$^+$ parent strain EAEP289 (Table 2).

TABLE 2. Resistance of *E. aerogenes* strains

Strain	Diam of growth inhibition (mm)[a] for drug:					
	CM	NFX	CIP	EM	TC[b]	DOX[b]
EAEP289, MDR	6	6	7	6	16	12
EAEP308(pDrive)	11	11	18	19	27	25
EAEP308(pEP867)	**6**	7	**10**	**10**	**20**	**16**
EAEP308(pLG339)	11	11	18	19	ND[c]	ND
EAEP308(pLGH-NSL26P)	**8**	11	18	**14**	ND	ND

[a] The diameter of the disks is 6 mm. CM, chloramphenicol (30 μg); NFX, norfloxacin (5 μg); CIP, ciprofloxacin (5 μg); EM, erythromycin (15 μg); TC, tetracycline (30 μg); DOX, doxycycline (30 μg). Boldface indicates values significantly different from control values.

[b] The influence of H-NSL26P on tetracycline antibiotic resistance could not be assessed as pLG339 carries a *tet* gene.

[c] ND, not determined.

Inner membrane extracts of EAEP308(pEP867) and EAEP308(pDrive) were analyzed by Western blotting with antibodies raised against *E. aerogenes* AcrA. A protein of about 37 kDa was detected in the EAEP308(pEP867) inner membrane, but not in that of EAEP308(pDrive) (Fig. 1, lanes 2 and 3). This observation suggests that the cross-reactive protein is EefA and that EefA production from the chromosomal *eefABC* locus is nondetectable in EAEP308.

The *eefABC* operon is cryptic in laboratory growth conditions. A suicide plasmid bearing an *eefA*::*lacZ* transcriptional fusion was introduced into EAEP295 (BW16627 background), EAEP289 (MDR strain), and EAEP308 (Δ*acrA*) (15). Integration of the *eefA*::*lacZ* suicide plasmid at the *eef* locus was confirmed by Southern blot analysis. A very weak activity of the reporter fusion was detected in the three resulting strains grown in Luria broth (LB) at 37°C (data not shown). EAEP289 lacks AcrR, the repressor of the *acrAB* operon; thus, *acrAB* is overexpressed in this strain (29). The absence of AcrR did not induce *eefA*::*lacZ* expression, suggesting that AcrR is not a repressor of *eefABC*.

Since transcription of multidrug efflux pumps can be induced by the presence of a relevant substrate in the growth medium (19, 30), we tested the effect of various antibiotics, detergents,

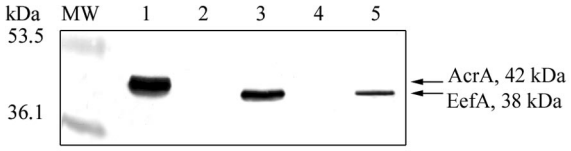

FIG. 1. Detection of EefA in *E. aerogenes* strains. Inner membrane proteins were prepared, and 10 μg was separated by SDS-PAGE on a 10% acrylamide gel, transferred to a nitrocellulose membrane, and immunodetected with antibodies raised against *E. aerogenes* AcrA. Lane 1, EAEP289; lane 2, EAEP308(pDrive); lane 3, EAEP308(pEP867); lane 4, EAEP308(pLG339); lane 5, EAEP308(pLGH-NSL26P).

bile salts, heavy metal salts, solvents, and dyes on the expression level of the *eefA*::*lacZ* fusion in EAEP60 (BW16627 background). We also varied temperature, osmolarity, pH, and O_2 growth conditions. We found no agent or condition that led to a detectable induction of the reporter fusion (data not shown).

In *E. aerogenes*, the overexpression of the global regulator MarA or RamA induces an MDR phenotype associated with an increase in AcrA production (7, 8). To decipher whether these efflux activators control *eef* expression, EAEP60 was transformed with multicopy plasmids bearing *marA* or *ramA*. We detected no induction of the reporter fusion when either MarA or RamA was overexpressed (data not shown), suggesting that the *eef* operon is not part of the MarA and RamA regulatory pathways or is strongly silenced by an upstream repressor.

H-NS is a repressor of *eefABC*. In *E. coli*, H-NS represses the expression of some multidrug efflux genes and deletion of *hns* confers MDR to an *acrAB*-deficient strain (25). We monitored the *eef* expression in an *E. coli hns* mutant by using a P*eef*::*lacZ* reporter plasmid. The 0.6-kb intergenic DNA region upstream of *eefABC* (P*eef*) was PCR amplified by using the primers *Bam*HI-P*eef*1 (5′ GGA-TCC-TTG-CGT-TTG-GCG-ATA-AGC 3′) and *Bam*HI-P*eef*2 (5′ GGA-TCC-TGA-GCG-AGG-CGG-TAG-TGC 3′) and *E. aerogenes* BW16627 genomic DNA as the template. The PCR product was cloned into pGEM-T to obtain pEP137. Digestion of pEP137 with BamHI released the 0.6-kb fragment, which was cloned into the BamHI site of pFus2-K to obtain pMM38, in which P*eef* controls *lacZ* expression. The P*eef*::*lacZ* expression level increased threefold in the *hns* mutant compared to the parental strain (Fig. 2). In addition, the P*eef*::*lacZ* expression level decreased upon transformation of the *hns* mutant with a plasmid bearing a wild-type *E. coli hns* copy. These results suggest that H-NS silences P*eef* activity in the heterologous host *E. coli*.

To evaluate the role of H-NS in *eef* regulation in *E. aerogenes*, we transformed EAEP308 Δ*acrA* with pLGH-NSL26P, which carries a dominant negative *E. coli hns* allele (35). Resistance to chloramphenicol and erythromycin was increased in EAEP308(pLGH-NSL26P) compared to EAEP308(pLG339) (Table 2). However, resistance to fluoroquinolones was not affected. Fluoroquinolones diffuse very efficiently across the bacterial membranes, so it is possible that the fluoroquinolone efflux is too slow to counterbalance entry. To assess EefA production, we analyzed inner membrane extracts of EAEP308(pLGH-NSL26P) and EAEP308(pLG339) by immunoblotting with antibodies raised against *E. aerogenes* AcrA. A single 37-kDa cross-reactive protein was detected in the inner membrane of the EAEP308

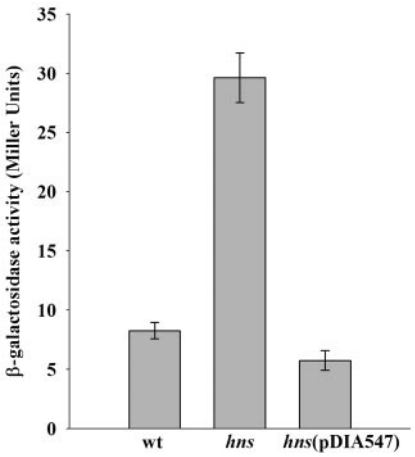

FIG. 2. Effect of H-NS on the expression of P*eef*::*lacZ* in *E. coli*. Histograms show β-galactosidase activity in *E. coli* PS2209 (wt), PS2652 (*hns*), and PS2652(pDIA547) carrying pMM38. β-Galactosidase was assayed on cells cultured overnight (21). The data are expressed as the means of a minimum of three independent experiments. Standard deviations were calculated.

expressing the dominant negative H-NSL26P but not in that of EAEP308(pLG339) (Fig. 1, lanes 4 and 5). Its expression level was 75% lower than that in EAEP308(pEP867) (Fig. 1, lanes 3 and 5). To identify this protein, whole-cell membranes of EAEP308 harboring pLG339 or pLGH-NSL26P were prepared and analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The first dimension was carried out on 7-cm isoelectrofocusing strips, pH 3 to 10 NL (Amersham Biosciences), in buffer containing 8 M urea, 2% Triton X-100, 10 mM dithiothreitol (DTT), and 0.5% IPG buffer, pH 3 to 10 (Amersham Biosciences, Uppsala, Sweden). The second dimension was carried out on 12.5% SDS-polyacrylamide slab gels. Proteins were either visualized with colloidal Coomassie brilliant blue staining (Fig. 3A) or transferred to a nitrocellulose membrane and immunodetected with antibodies raised against *E. aerogenes* AcrA (Fig. 3B). Two immunoreactive

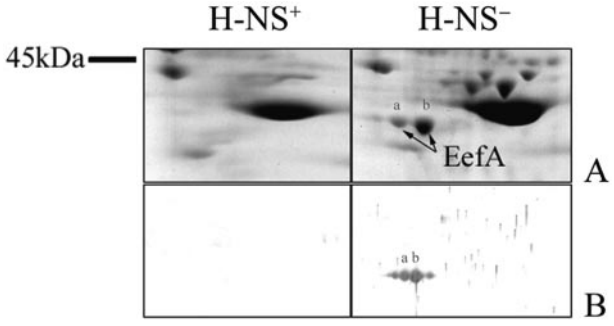

FIG. 3. 2D-PAGE analysis of the membrane protein profiles from EAEP308(pLG339) H-NS$^+$ and its H-NS$^-$ derivative expressing *E. coli* dominant negative H-NSL26P. Equal amounts of proteins (up to 100 μg) from strains to be compared were separated by 2D-PAGE. Proteins were either visualized with colloidal Coomassie brilliant blue staining (A) or transferred to a nitrocellulose membrane and immunodetected with antibodies raised against *E. aerogenes* AcrA (B). Only the region in the vicinity of the EefA spots is shown. EefA isoforms a and b are indicated by arrowheads.

spots (a and b in Fig. 3) were excised from the gel, in gel tryptic digested, and analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (32). In EAEP308(pLGH-NSL26P), an immunoreactive protein was resolved as a major spot of pI 5.3 and an apparent mass of 37 kDa, in agreement with the theoretical values computed from the EefA amino acid sequence. This protein was undetectable in extracts of EAEP308(pLG339) (Fig. 3). The peptide masses were used to search in sequence databases. The MALDI-TOF analysis of tryptic peptides from spots a and b accounted for 28 and 65% coverage of the *E. aerogenes* EefA precursor sequence tr Q8GC84, respectively, and the matching peptides were distributed throughout the entire sequence (data not shown).

Conclusions. Numerous H-NS target genes are involved in bacterial adaptation to stressful environmental conditions and virulence (3, 14). The biological relevance of the *eef* operon silencing is not known. However, like other commensal or pathogenic bacteria, *E. aerogenes* has to orchestrate drastic changes in its gene expression profile in order to adapt to the host-associated conditions. Further studies might decipher the regulation and physiological role of the *eef* operon.

Nucleotide sequence accession number. The nucleotide sequences of the *E. aerogenes eefABC* operon and flanking genes *regR* (*orf 1*), *act* (*orf 2*), and *yfeU* (*orf 6*) have been deposited in the GenBank database under accession number AJ508047.

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