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Physiological characterisation of the efflux pump system of antibiotic-susceptible and multidrug-resistant *Enterobacter aerogenes*

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ABSTRACT

Enterobacter aerogenes predominates among Enterobacteriaceae species that are increasingly reported as producers of extended-spectrum β -lactamases. Although this mechanism of resistance to β -lactams is important, other mechanisms bestowing a multidrug-resistant (MDR) phenotype in this species are now well documented. Among these mechanisms is the overexpression of efflux pumps that extrude structurally unrelated antibiotics prior to their reaching their targets. Interestingly, although knowledge of the genetic background behind efflux pumps is rapidly advancing, few studies assess the physiological nature of the overall efflux pump system of this, or for that matter any other, bacterium. The study reported here evaluates physiologically the efflux pump system of an *E. aerogenes* ATCC reference as well as two strains whose MDR phenotypes are mediated by overexpressed efflux pumps. The activities of the efflux pumps in these strains are modulated by pH and glucose, although the effects of the latter are essentially restricted to pH 8, suggesting the presence of two general efflux pump systems, i.e. proton-motive force-dependent and ABC transporter types, respectively.

1. Introduction

The advent of multidrug resistance in Gram-negative bacteria has increased the range of infections owing to the problematic nature of therapy. Consequently, infections by *Enterobacter aerogenes* that were once less frequent are now becoming increasingly more frequent in the hospital environment [1]. As an example, two clones of ceftazidime-resistant *E. aerogenes* have been spreading throughout Belgian hospitals to the point that they have become a serious healthcare issue both in hospitals and in the community [2]. Moreover, strains of *E. aerogenes* now account for >50% of all Gram-negative organisms that produce extended-spectrum β -lactamases (ESBLs) and, inasmuch as ESBLs are the major cause of treatment failure, the opportunity for the development of multidrug-resistant (MDR) phenotypes has resulted [3]. Among the mechanisms that contribute to a MDR phenotype in *E. aerogenes* is overexpression of genes that regulate and code for efflux pumps which extrude unrelated antibiotics before they reach their intended targets [4]. Interestingly, whilst the appearance of multidrug resistance in this species has been essentially made possible by the production and release of type C β -lactamases, the existence of an overexpressed efflux pump further contributes to the progressive evolution of multidrug resistance [4–6].

The MDR phenotype of *E. aerogenes* coupled with its ESBL-producing capacity has resulted in increased emergence of this pathogen in hospital units [7–9] and

in the description by the media as ‘killer bugs’ or ‘superbugs’. Indeed, the infections they cause result in a high cost in terms of morbidity, treatment failures and subsequent mortality [2]. Although at the time of this writing no agents are available for inhibition of type C β -lactamases, a variety of agents have been identified as having the potential to inhibit overexpressed efflux pumps of Gram-negative bacteria [10]. However, although some of these inhibitors of efflux pumps affect the function of the pump itself [efflux pump inhibitors (EPIs)] [11,12], there is now evidence that some EPIs may have an effect on regulatory genes involved in the control of efflux expression and genes that code for transporters [13,14]. Because of this, assessment of EPIs must also include their potential genetic actions. Nevertheless, although the genetic basis for an overexpressed efflux pump is essential, the actual function of an efflux pump need not be compatible with its level of genetic expression and regulation [15]. As an example, in *Escherichia coli* strains whereas transcription of the *acrEF* gene is 80-fold greater in a tetracycline-resistant strain than that of its parental tetracycline-susceptible strain, its level of resistance is equal to that of the tetracycline-resistant strain whose *acrAB* is transcribed only 6-fold above that of its parent [15].

With the exception of some early demonstrations of efflux pumps as well as a recent report [16], assessment of the function of specific efflux pumps is essentially absent from the literature. Understanding that resistance–nodulation–cell division (RND) tripartite efflux pumps derive their energy for function from the

membrane Δp (potential gradient, $\Delta p = \Delta \phi + RT \log \Delta pH$, where $\Delta \phi$ is the difference in electrochemical gradient across the membrane, RT is the amount of ATP harnessed at 37 °C and ΔpH is the difference in pH across the membrane) (proton-motive force across inner membrane) suggests that the efficacy of these pumps would be best at low pH and less so at pH >7 given the paucity of protons at the latter pH. At pH >7 one would suppose that ATP-binding cassette (ABC)-type transporters would derive their energy from the hydrolysis of ATP, inasmuch as such hydrolysis would be favoured by the $F_0 - F_1$ energy system of the cell [17]. Moreover, because the dissociation constant (K_d) for substrates bound by the AcrB transporter is low at high pH and high at low pH [18], the dissociation of a substrate such as ethidium bromide (EB) from the AcrB transporter could be sufficiently slow at physiological pH 7 to render the AcrAB efflux pump somewhat limited if the organism is challenged with a noxious substrate that it must extrude for its survival [19]. Given these considerations, the described study here has assessed the role of pH in the activity of the efflux pump system of the intrinsically susceptible *E. aerogenes* ATCC reference strain as well as two MDR phenotypic strains whose resistance is due to an overexpressed efflux pump system.

2. Materials and methods

2.1. Materials

Mueller–Hinton (MH) in powder form was used for the preparation of MH broth and MH agar. Antibiotics in powder form were purchased from Difco Laboratories (Detroit, MI). EB, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and Phe-Arg- β -naphthylamide (PA β N) were purchased from Sigma-Aldrich Química SA (Madrid, Spain).

2.2. Bacteria

Antibiotic-susceptible *E. aerogenes* ATCC 13048 and MDR *E. aerogenes* EA27 and CM64 strains have been previously fully characterised [6,20,21]. The MDR strains are resistant to norfloxacin, chloramphenicol, tetracycline and other antibiotics and overexpress the AcrAB-TolC efflux pump [6,20,21].

2.3. Methods

2.3.1. Minimum inhibitory concentrations (MICs)

The MIC of antibiotics, CCCP and PA β N against the strains employed in this study were determined in MH medium adjusted to pH 5, 7 and 8 by the microbroth dilution method according to Clinical and Laboratory Standards Institute guidelines [22].

2.3.2. Assessment of putative efflux of ethidium bromide by the ethidium bromide agar method

Assessment of efflux by the EB agar method has been previously described in detail [23,24]. Briefly, plates of MH agar of pH 5, 7 and 8 containing increasing concentrations of EB were swabbed with each bacterial strain as spokes of a wheel (cartwheel manner) and the plates were incubated for 18 h at 37 °C, examined under ultraviolet light and photographed using a GEL DOC XR instrument (Bio-Rad Laboratories, Hercules, CA). The minimum concentration of EB that produces fluorescence indicates that the amount of EB that the cell can extrude has been exceeded and hence EB accumulates within the cell.

2.3.3. Assessment of efflux pump activity

Assessment of efflux pump activity was conducted by the automated EB method as described previously [25]. This method affords a real-time estimation of accumulation of EB and its efflux. Briefly, strains were cultured in MH medium until they reached an optical density at 600 nm (OD_{600}) of 0.6 and centrifuged at 13 000 rpm for 3 min. The pellets were re-suspended in saline buffer, the OD_{600} adjusted to 0.6 and 1 mL aliquots were transferred to microtubes. The microtubes were centrifuged and the pellets were re-suspended in saline buffer at pH 5, 7 or 8. Aliquots of 0.045 mL were transferred to microtubes of 0.1 mL and then 0.045 mL of EB in glucose-free saline of pH 5, 7 and 8 was added to

the respective tubes of the same pH. Because metabolic energy is required for efflux of EB, the absence of glucose ensures sufficient accumulation of the agent. The final concentration of EB for all experiments was 1.0 mg/L. As concentrations of EB much greater than 1 mg/L exceed the ability of the cell to extrude the agent, the level of intracellular agent rapidly increases and results in its intercalation between the nucleic bases of DNA. EB when bound to DNA is no longer available for extrusion [26]. The tubes were rapidly transferred to a Corbett 3000 thermocycler (Qiagen, Doncaster, Australia) programmed with cycles of 1 min at a constant temperature of 37 °C. The total average time from addition of the EB–saline to as many as 12 individual tubes to the transfer of the tubes to the instrument and re-starting the instrument did not exceed 60 s. Accumulation of EB in each tube was followed on a real-time basis by assessment of fluorescence emitted. Excitation and emission wavelengths were 535 nm and 585 nm, respectively. Whereas the medium containing 1.0 mg/L of EB does not appreciably fluoresce, as the concentration of EB builds up in the periplasm of the Gram-negative bacterium, fluorescence is readily detected by the instrument [23]. Accumulation of EB at various pH levels was followed for up to 25 min.

Assessment of efflux of EB at pH 5, 7 and 8 was conducted as follows.

Accumulation of EB at pH 5, 7 and 8 as described above was first conducted for up to 25 min, after which time the instrument was stopped and 0.010 mL of saline at pH 5, 7 and 8 either lacking or containing glucose to yield a concentration of

0.4% was added to the respective tubes. The tubes were then transferred to the instrument and the instrument was re-started. The total amount of time for this addition did not exceed 2.5 min. Fluorescence was followed for a minimum period of 10 min.

The effects of varying concentrations of CCCP and PA β N on efflux of EB was carried out as follows. Accumulation of EB in glucose-free saline of pH 5, 7 and 8 was conducted as described and when the plateau of accumulation was reached the instrument was stopped and 0.045 mL of glucose-saline of pH 5, 7 and 8 containing varying concentrations of CCCP and PA β N was added to the saline-containing tubes at the respective pH. The tubes were placed into the instrument, the instrument was re-started and fluorescence was followed for up to 15 min. The total time for these additions did not exceed 2.5 min. For some experiments, addition of CCCP at varying concentrations was in matched-pH glucose-free medium. This component of the experiment afforded an additional control that would define any role of metabolic energy in conjunction with any effect produced by given concentrations of CCCP on efflux of EB and modulation of efflux at a given pH. The concentrations of CCCP and PA β N were $<0.25\times$ MIC and had no effect on the viability of the *E. aerogenes* strains employed in this study.

3. Results

The MICs of various antibiotics against the *E. aerogenes* strains used in this study are presented in Table 1. As noted in the table, the CM64 and EA27 strains that overexpress the AcrAB efflux pump exhibit a MDR phenotype. Evaluation of the effect of agents for inhibitory activity against efflux requires that the agent be used at a concentration that has no significant effect on the viability and growth rate of the bacterium [29]. Consequently, the MICs of EB, CCCP and PA β N against each of the *E. aerogenes* strains were also determined in MH broth at pH 5 and 8, thus allowing the selection of concentrations that had no effect on the viability of the strains employed in this study (data not shown).

The effect of pH on retention of EB by the *E. aerogenes* strains is illustrated in Fig. 1. Whereas at pH 8 fluorescence is detected for all of the strains at 0.5 mg/L EB, at pH 5 the CM64 and EA27 strains do not fluoresce even with a concentration as high as 2.5 mg/L EB. Unlike these latter MDR strains, the ATCC 13048 strain begins to fluoresces with 0.2 mg/L EB at pH 5 (data not shown). These results collectively suggest that: (i) the viability of the organism is not affected by concentrations as high as 600 mg/L EB; (ii) permeability of the strains to EB at pH 8 is sufficient with a concentration of EB as low as 0.5 mg/L; (iii) the strains are able to extrude EB given the difference observed between ATCC and resistant strains; and (iv) the MDR strains EA27 and CM64 have an

overexpressed efflux pump system that is readily evident at pH 5 compared with that present in the ATCC 13048 strain [4,5,27].

The effect of pH and glucose on the accumulation of EB by the strains was evaluated by the automated EB method [19]. As shown in Fig. 2, accumulation of EB by the ATCC 13048 strain is not affected by pH unless glucose is absent from the medium, and only at pH 8. Similar results were obtained with the MDR strains EA27 and CM64 (data not shown). These results suggest that at pH 8 extrusion of EB is markedly affected by the absence of glucose, as would be expected if the efflux pump system at this pH was dependent on the membrane energy conferred by glucose metabolism (hydrolysis of ATP). Because no accumulation per unit period of time takes place at pH 5 where the Δ pH is strong [19], intracellular concentration of EB is dependent only on the availability of protons and is independent of metabolic energy (glucose). The accumulation assays without glucose at pH 5 and 8 were repeated and after 25 min the instrument was put into its pause mode and pH-matched saline containing glucose to yield a final concentration of 0.4% was added and the instrument re-started. As shown in Fig. 3, addition of glucose at pH 8 to the ATTC 13048- and CM64-containing tubes immediately causes a drop in fluorescence from 10 to 25 fluorescence units compared with the control without glucose. This drop in fluorescence is maintained for the duration of the assay. At pH 5, glucose has no effect. These results demonstrate that at pH 8 efflux is dependent upon a source of metabolic energy (glucose).

To determine that the degree of fluorescence reflective of accumulation of EB is indeed due to an efflux pump system, the EB accumulation assay was repeated with glucose-free saline at pH 5 and 8 for each of the *E. aerogenes* strains. After the instrument was put into pause mode, varying concentrations of CCCP, an uncoupler of membrane energy, in glucose-containing saline of pH 5 and 8 were added, the instrument was re-started and the amount of relative fluorescence was followed for an additional 10 min. Addition of pH-matched saline with and without glucose served as controls. The data presented in Fig. 4 are for addition of 5 mg/L CCCP. As is evident from Fig. 4, addition of CCCP at pH 5 causes an increase in the amount of fluorescence due to EB retained by the ATCC 13048 strain and a considerably lower increase for the two MDR strains EA27 and CM64. At pH 8, addition of CCCP markedly increases accumulation of EB in the ATCC 13048 and EA27 strains to the same level but does not affect the accumulation of EB by the CM64 strain, suggesting that at pH 8 the extrusion of EB is taking place by an ABC transporter that receives its energy directly from the hydrolysis of ATP [19].

The smaller effect of CCCP on the accumulation of EB at pH 5 noted for the ATCC and EA27 strains is interpreted to be due to the large concentration of surface protons [19,30] that exceed the binding capacity of CCCP. At pH 8, the proton concentration is rapidly bound such that the antiport proton-dependent efflux pump system of the ATCC 13048 and EA27 strains is denied the required

energy for its action. At pH 8 any contribution made by the presence of metabolic energy is masked by the acute accumulation of EB produced by CCCP. Unlike the effects of CCCP on the ATCC 13048 and EA27 strains, addition of CCCP has no effect on the accumulation of EB by the CM64 strain.

PA β N has been repeatedly described as an EPI [31,32]. However, our recent results suggest that PA β N has no effect on efflux but has an effect on accumulation. The effect of PA β N on intracellular concentration is not due to direct inhibition of efflux pump activity but rather to competition with EB transport [19,24,25]. Nevertheless, we first assessed PA β N for any effect on efflux, and hence accumulation, of EB by each of the strains in glucose-free medium at pH 5 and 8; after 25 min, 20 mg/L and 40 mg/L PA β N in glucose-containing medium was added. As shown in Fig. 5, addition of PA β N in glucose-containing saline at pH 5 and 8 has no effect on accumulation of EB by any of the *E. aerogenes* strains, even with a concentration as high as 40 mg/L. The additions contained glucose to ensure that the metabolic energy required for efflux at pH 8 was readily available.

4. Discussion

The two overexpressing strains studied in this work differ in terms of overexpression of efflux pumps, mutations and porin expressions [21,27]. According to Ghisalberti et al. [21], strain CM64, which became resistant

following prolonged exposure to increasing concentrations of chloramphenicol, presents overexpression of the AcrAB-TolC efflux pump. However, strain EA27, a clinical isolate also resistant to chloramphenicol, was reported as having a deletion in the *acrR* gene that induces upregulation of the *acrAB* efflux pump operon as well as exhibiting lower expression of Omp36, the major non-specific porin of *E. aerogenes* [21]. In contrast to EA27, the opposite is true for the CM64 and the parental strains [21,27]. These differences in resistance mechanisms adopted by the two MDR strains were also visible in our work. CM64 shows resistance mechanisms dependent on pH and glucose availability. On the other hand, these effects are not evident for the EA27 and ATCC strains. Because the mechanisms of resistance of the CM64 strain are based on overexpression of the AcrAB efflux pump, extrusion of EB is mediated by proton changes obtained directly from the transmembrane proton gradient. At pH 8, owing to slow dissociation of the EB substrate from the AcrB transporter [18], efflux by the RND AcrAB efflux pump may be limited. It is at this pH 8 where the need for metabolic energy (glucose) is evident, suggesting the involvement of a putative ABC transporter [19].

At neutral pH, the energy derived from glucose or other sources is used to export cytoplasmic H⁺ to the periplasmic space where it is used by the AcrB pump for drug antiport activity. Addition of CCCP collapses the energy of proton transport to the periplasm, reducing the H⁺ concentration within the periplasm and consequently altering AcrB function. At acidic pH, the proton periplasmic

concentration is high and CCCP cannot downregulate the activity of the AcrB pump.

PA β N is again shown not to have any effect on EB efflux by Gram-negative bacteria [19,23,25], confirming that the mechanism by which PA β N reduces efflux-mediated resistance to a given antibiotic is due to competition between PA β N and EB [33]. The degree of competition has recently been shown to be in favour of extrusion of PA β N, resulting in a Henri–Michaelis–Menten constant (K_M) of 4.21 mg/L versus 1 mg/L EB by the efflux machinery of *E. coli* [19]. Lastly, because dissociation of an AcrB substrate is very fast at low pH [18], if a true inhibitor of an efflux pump is to be designed its activity must be assessed at pH 5 to ensure that it truly binds and inhibits the RND efflux pump of Gram-negative bacteria.

It is also interesting to mention that PA β N is able to increase significantly, at neutral pH, the intracellular accumulation of chloramphenicol in the *E. aerogenes* strains tested [28]. This increase suggests that PA β N and chloramphenicol may use the same affinity sites in the AcrB cavity, in contrast to EB. Consequently, PA β N may successfully compete for extrusion with chloramphenicol as previously reported and partially restore chloramphenicol susceptibility in different efflux-mediated resistant strains [19]. Alternatively, EB may use a

parallel efflux pump during the first step before reaching the central AcrB cavity that is open to the TolC exit canal [34].

At this moment, it is important to note that *E. aerogenes*, like *E. coli*, is a commensal bacterium of the digestive tract where it faces an acidic pH that consequently may provide sufficient periplasmic proton concentration to drive the proton antiport necessary for drug extrusion.

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Competing interests

None declared.

Ethical approval

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Fig. 1. Effect of pH on the accumulation of ethidium bromide (EB) by strains of *Enterobacter aerogenes*.

Fig. 2. Effects of pH and glucose (glu) on the accumulation of ethidium bromide by *Enterobacter aerogenes* ATCC 13048.

Fig. 3. Effect of glucose (glu) on efflux of ethidium bromide at pH 5 and 8 by (A) *Enterobacter aerogenes* ATCC 13048 and (B) *E. aerogenes* CM64 strain.

Fig. 4. Effect of 5 mg/L carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on efflux of ethidium bromide at pH 5 and pH 8 by *Enterobacter aerogenes*: (A1) *E. aerogenes* ATCC 13048, pH 5; (A2) *E. aerogenes* ATCC 13048, pH 8; (B1) *E. aerogenes* EA27, pH 5; (B2) *E. aerogenes* EA27, pH 8; (C1) *E. aerogenes* CM64, pH 5; and (C2) *E. aerogenes* CM64, pH 8. glu, glucose.

Fig. 5. Effect of 40 mg/L Phe-Arg- β -naphthylamide (PA β N) on efflux of ethidium bromide at pH 5 and pH 8 by *Enterobacter aerogenes*: (A1) *E. aerogenes* ATCC 13048, pH 5; (A2) *E. aerogenes* ATCC 13048, pH 8; (B1) *E. aerogenes* EA27, pH 5; (B2) *E. aerogenes* EA27, pH 8; (C1) *E. aerogenes* CM64, pH 5; and (C2) *E. aerogenes* CM64, pH 8. glu, glucose.

Table 1

Antimicrobial susceptibilities of the *Enterobacter aerogenes* strains studied

[27,28]

	MIC (mg/L)		
	ATCC 13048	CM 64 ^a	EA 27 ^b
Cefepime	0.125	0.5	64/128
Imipenem	0.25	0.25	8
Ceftazidime	0.5	1	512
Chloramphenicol	8	512	512/1024
Tetracycline	2	16	16
Nalidixic acid	8	128	512
Norfloxacin	0.5	4	256
Ciprofloxacin	0.125	1	32
Ofloxacin	0.5	N/D	>128
Sparfloxacin	0.125	1	256
Erythromycin	128	512	N/D
Gentamicin	1	1	2

MIC, minimum inhibitory concentration; N/D, not determined.

^a CM 64 strain overexpresses the AcrAB-TolC efflux pump [21,27].

^b EA27, a clinical isolate, is resistant to chloramphenicol and has a deletion in the *acrR* gene that induces upregulation of the *acrAB* efflux pump operon as well as exhibiting reduced expression of Omp36, the major non-specific porin of *E. aerogenes* [21].

Legend:

crip