SHORT COMMUNICATION

Abrogation of the resistance of choline-induced *Pseudomonas aeruginosa* virulence to sub-MIC erythromycin by ethanol

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Despite *Pseudomonas aeruginosa* antibiotic resistance, erythromycin (ERM, a macrolide) at subinhibitory concentration (sub-MIC) reduces its pathogenicity. We assessed ERM effects on *P. aeruginosa* in cultures containing choline (Ch) without and with 1% ethanol (Et) addition. Ch, as an osmoprotectant, increases the following virulence factors (VIFs): lectins (haemagglutination); proteases (casein and elastin lysis); haemolytic phospholipase C (PLC-H; haemolysis); pyocyanin (pigment o.d.) and autoinducers (violacein bioassay). Ethanol also increases lectins, proteases, pyocyanin, autoinducers and rhamnolipid (RHAL; haemolysis) formation, but reduces Ch-induced PLC and protease (elastase) activities. ERM has been shown to totally suppress the Et-induced VIFs, whereas partially reducing the Ch-induced ones. Unexpectedly, ERM combination with 1% Et dramatically annuls the Ch-induced factors. Et contribution might be attributed to its effect on cell membrane, displaying synergism with ERM, whereas antagonizing Ch osmoprotective potential and shifting gene expression. This information is worth further molecular investigation and clinical consideration for skin infection therapy.

The ISME Journal (2008) 2, 1243–1246; doi:10.1038/ismej.2008.79; published online 28 August 2008

Subject Category: microbe-microbe and microbe-host interactions

Keywords: alcohol; choline osmoprotection; macrolide sub-MIC; *Pseudomonas aeruginosa*; virulence factors

Introduction

Infections by the opportunistic pathogen *Pseudomonas aeruginosa*, causing nosocomial morbidity and mortality in cystic fibrosis, bronchiolitis, AIDS and burn patients are resistant to antibiotics. Nevertheless, an important improvement in patient health has been attained by long-term (years) treatment with erythromycin (ERM) or other macrolides at subinhibitory concentration (sub-MIC). This treatment reduces virulence without bacterial eradication (Fujii *et al.*, 1995; Kudoh *et al.*, 1998). Therefore, it has been suggested that at sub-MIC, ERM does not inhibit protein synthesis in exponentially growing cells (by binding to 50S ribosomal subunit), but functions in the stationary phase following the gene expression shift (Wagner *et al.*, 2006). Its interaction with divalent cations that cross-bridge lipopolysaccharides, destabilizes bac-

terial cell outer membrane, increasing permeability (Imamura *et al.*, 2005). *In vitro* studies have indicated that macrolide sub-MIC reduces expression of *P. aeruginosa* exotoxins, exoenzymes (Kita *et al.*, 1991) and lectins (galactophilic PA-IL and fucose-binding PA-III (Gilboa-Garber, 1982; Imberty *et al.*, 2004), which mediate *P. aeruginosa* biofilm formation (Tielker *et al.*, 2005; Diggle *et al.*, 2006) and adhesion to target cells for infection establishment (Gilboa-Garber and Garber, 1989)), impairing biofilm formation (Kobayashi, 1995). It also diminishes haemolysins (phospholipase C (PLC-H) and rhamnolipids (RHALs)), pyocyanin and 'quorum-sensing autoinducers' (HSLs), which coregulate virulence factor (VIF) formation (Winzer *et al.*, 2000) (Sofer *et al.*, 1999) and affect the host cells (Wagner *et al.*, 2006).

The aim of this study is to examine the ERM sub-MIC effects on some *P. aeruginosa* VIFs in presence of choline (Ch) without and with 1% ethanol (Et), as both of them augment *P. aeruginosa* VIF formation. Ch augments cholinesterase (Gilboa-Garber *et al.*, 1973), lectins (Gilboa-Garber, 1982), haemolytic phospholipase (Sage and Vasil, 1997), proteases, and HSLs (Katri and Gilboa-Garber, 2007), and Et increases alginate (DeVault *et al.*, 1990), RHALs

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Received 2 January 2008; revised 22 June 2008; accepted 23 July 2008; published online 28 August 2008

(Matsufuji *et al.*, 1997), proteases, pyocyanin, lectins and HSLs (Katri and Gilboa-Garber, 2007).

Materials and methods

Pseudomonas aeruginosa

ATCC 33347 cultures were grown in Eagon–Grelet medium without and with Ch (0.2%, daily), Et (1%) or both (Katri and Gilboa-Garber, 2007). ERM was added to them at sub-MIC ($50 \mu\text{g ml}^{-1}$). The cultures were vigorously (providing aeration) shaken at 28°C for 3 days for attaining the highest lectin, protease, elastase and haemolysin levels (Gilboa-Garber, 1982; Katri and Gilboa-Garber, 2007). Washed cell pellets were kept at 4°C until disintegration on the same or next day (by ultrasonic treatment, at 10% (w/v suspension)). The cell-free extracts (used for lectin

assays) and culture supernatants (used for all the other activities) were kept at -20°C .

Lectins were assayed by differential haemagglutination test (PA-IL with mannose and PA-IIL with galactose (Gilboa-Garber, 1982; Katri and Gilboa-Garber, 2007)). Haemagglutination titre was represented by Log_2 per dilution.

Proteolysis was assayed at 37°C using skim milk (2.5%) in agar (2%) plates incubated for 24 h (Katri and Gilboa-Garber, 2007), azocasein in tubes incubated for 30 min (stopped by TCA) (Jensen *et al.*, 1980), and elastin Congo red in tubes incubated for 2 h (stopped by EDTA). The clear zones around the wells in the skim milk plates were measured (mm^2) and the released azo and Congo red colours were determined at 370 and 495 nm, respectively.

Figure 1 Levels of lectins (PA-IL and PA-IIL), proteases (Pr), pyocyanin (Py), haemolytic phospholipase C (PLC-H), rhamnolipids (RHALs) and short-chain autoinducers (HSLs) in EG medium without and with choline (Ch; 0.2% added daily), ethanol (Et; 1%) and erythromycin (a macrolide) at subinhibitory concentration (ERM sub-MIC; $50 \mu\text{g ml}^{-1}$). The data represent means of 5–7 experimental results + s.e.m. The Y-axis represents virulence factor (VIF) relative levels (related to the respective asterisked (= 10) values, except PA-IL and PA-IIL haemagglutination titres, which are represented by Log_2 per dilution). The calculations of all the activities were based on combining mean values of the results obtained using the different methods described in the Materials and methods and taking the cell mass into account. *Denotes the 10-value column.

Pyocyanin green-blue pigment was determined, following shaking (for oxidation), at 660 nm (Katri and Gilboa-Garber, 2007), and its water-soluble pink product (following chloroform extraction and acidification by 1 N HCl) was determined at 520 nm (Essar et al., 1990).

Phospholipase C haemolysis and RHALs were assayed at 37 °C using 2.5% (v/v) thrice-washed erythrocyte suspension in saline both in 2% agar plates and in tubes. These were incubated for 24 and 2 h, respectively, before measuring clear zones around the sample-containing wells (Katri and Gilboa-Garber, 2007) and released haemoglobin O.D. at 540 nm, respectively. Discrimination between heat-sensitive PLC-H and heat-stable RHAL haemolytic activities was attained by overnight dialysis against saline (removing only RHALs of low molecular weight) and by 10-min boiling (inactivating only PLC-H). Net PLC-H activity was also calculated by subtracting the RHAL (boiled) from the total haemolytic activity.

Short-chain autoinducer (including C₄-HSL, C₆-HSL and 3-oxo-C₆-HSL) levels were recorded (without individual identification) following extraction and TLC separation, by bioassay, exploiting *Chromobacterium violaceum* CVO26 mutant (kindly donated by Professor P Williams, Nottingham) that forms a dark purple pigment violacein in presence of short autoinducers, according to their levels (McClellan et al., 1997).

Results and discussion

Reports on ERM sub-MIC efficiency in reducing *P. aeruginosa* VIFs *in vivo* (Fujii et al., 1995; Kudoh et al., 1998) and *in vitro* (Kita et al., 1991; Kobayashi, 1995; Sofer et al., 1999) led us to examine its efficacy in presence of the osmoprotectant Ch (which is released in tissues damaged by *P. aeruginosa* (Chen et al., 2005)) and of Et, both of which increase *P. aeruginosa* VIF formation. *P. aeruginosa* cultures were grown for 3 days to obtain peak levels of lectins (Gilboa-Garber, 1982), proteases and haemolysins, and post-peak pyocyanin and HSL activities (Katri and Gilboa-Garber, 2007).

Figure 1 (2a and 3a) shows that both Ch and Et stimulate *P. aeruginosa* production of lectins (both PA-IL and PA-III), proteases, pyocyanin, HSL and haemolysins (PLC-H and RHALs, respectively). A combination of the two leads to a general increase of VIF levels, excluding reduction in proteolytic (mainly elastase) and PLC-H activities (Figure 1: 4a). ERM sub-MIC suppresses the Et-induced activities (Figure 1: 3b), but reduces only a few (PA-III and pyocyanin) of the Ch-induced ones (Figure 1: 2b). Ch-induced PA-IL, protease, PLC-H and HSL activities are not repressed by ERM (in Et absence) and some of them even slightly increase (Figure 1: 2a and 2b). Therefore, it was surprising to find that ERM-Et combination completely abolished all the

Ch-induced VIFs (Figure 1: 4b), resembling the Et-induced ones, by leaving only constitutive PLC-H activity.

The mechanism underlying the reinforcement of ERM repression of the osmoprotective Ch-induced *P. aeruginosa* VIFs might be owing to the Et effect on bacterial cell membrane permeability, synergistically increasing ERM efficiency, while diminishing Ch osmoprotective potential, and shifting gene expression from the Ch-induced ERM-resistant activities to Et-induced ERM-sensitive ones. As low Et concentrations are applied harmlessly in treating skin infections (iodine and other antiseptic tinctures), the addition of 1% Et to the treatment of skin infections by antibiotics might be considered (depending on *in vivo* examinations).

Acknowledgements

This study is a part of N Katri's (née Melamed) PhD thesis at Bar-Ilan University, 2003. We thank Ms Sharon Victor and Ms Ela Gindy for their great help in the preparation of the manuscript and graphic presentation.

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