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Prolonged exposure of methicillin-resistant *Staphylococcus aureus* (MRSA) COL strain to increasing concentrations of oxacillin results in a multidrug-resistant phenotype

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Abstract

Our previous studies demonstrated that exposure of a bacterium to increasing concentrations of an antibiotic would increase resistance to that antibiotic as a consequence of activating efflux pumps. This study utilises the same approach; however, it employs the methicillin-resistant *Staphylococcus aureus* (MRSA) COL strain, which is highly resistant to oxacillin (OXA). MRSA COL was adapted to 3200 mg/L of OXA. Changes in resistance to other antibiotics were evaluated and efflux pump activity during the adaptation process was determined. MRSA COL was exposed to stepwise two-fold increases of OXA. At the end of each step, minimum inhibitory concentration determination for erythromycin (ERY) and other antibiotics was conducted. Reserpine (RES) was employed to evaluate whether resistance to ERY was dependent on efflux pump activity. Efflux pump activity was also evaluated using the ethidium bromide (EB) assay. DNA typing of the products of each culture step was conducted to assess purity. Serial exposure of MRSA COL to increasing concentrations of OXA resulted in increased resistance to ERY, which could be eliminated with RES. Evaluation of efflux pump activity by the EB method indicated increased efflux activity. Resistance to ERY was accompanied by resistance to kanamycin, amikacin, ofloxacin, norfloxacin, ciprofloxacin and rifampicin. This is the first time that a multidrug-resistant phenotype has been experimentally produced as a consequence of exposure of the organism to an antibiotic to which it is initially highly resistant.

Keywords: MRSA COL; Adaptation; Oxacillin; High concentration; MDR; Efflux pumps

1. Introduction

We have previously shown that exposure of *Escherichia coli* to stepwise increases in tetracycline (TET) concentrations increases resistance from 2.0 mg/L to >12 mg/L of TET [1]. Accompanying this induced resistance are significant increases in resistance to many other antibiotics and non-antibiotic agents. Hence, the process of exposure to increasing concentrations of one antibiotic produces a multidrug-resistant (MDR) phenotype. This MDR phenotype is accompanied by significant increased activity of genes that code for nine distinct transporter proteins [1]. Exposure of isoniazid (INH)-sensitive *Mycobacterium tuberculosis* to increasing concentrations of INH will also increase the resistance of the organism from 0.2 mg/L to >40 mg/L [2]. However, increased resistance to INH is not accompanied by resistance to any other drug employed for the therapy

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of pulmonary tuberculosis. If we assume that events at the level of the bacterial cell envelope that result in increased efflux pump activity are independent of a chromosomal mutation that bestows high-level resistance of the bacterium to a given antibiotic, then prolonged exposure of that bacterium to increasing concentrations of the antibiotic to which it is resistant may induce the appearance of a MDR type efflux pump. We have examined this possibility using the methicillin-resistant *Staphylococcus aureus* (MRSA) COL strain whose resistance to 400 mg/L oxacillin (OXA) is due to the acquired mecA element [3]. We exposed this strain to stepwise increases of OXA ranging from 50 mg/L to 6400 mg/L and at each level of increased resistance we examined the organism for any changes in its susceptibility to other antibiotics and for evidence of efflux activity.

2. Material and methods

2.1. Bacteria

The bacterium employed throughout this study was *S. aureus* COL strain, an early methicillin-resistant strain originally isolated in a hospital in Colindale, UK (generously provided by Prof. Dr Hermínia de Lencastre, Professor and Head, Laboratório de Genética Molecular, Instituto de Tecnologia Química e Biológica, Oeiras, Portugal). Characterisation of the COL strain was previously reported by Prof. Dr de Lencastre's group [4].

Trypticase soy (Difco, Madrid, Spain) was used to prepare trypticase soy broth (TSB) and trypticase soy agar.

2.2. Antibiotics and reagents

OXA, erythromycin (ERY) in powder form, ethidium bromide (EB) and reserpine (RES) were purchased from Sigma–Aldrich Química SA (Madrid, Spain). OXA and ERY were also provided as Kirby–Bauer disks of 1 μg and 15 μg, respectively, by Oxoid Ltd. (Basingstoke, UK).

2.3. Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

Determination of the MIC and MBC of antibiotics against *S. aureus* was conducted by the methods previously described [5,6].

2.4. Culture

MRSA COL was initially grown in TSB until it reached one-half of its maximum optical density (OD), as determined spectrophotometrically at 545 nm. An aliquot of 10 μL was transferred to 10 mL tubes containing 50 mg/L OXA in 10 mL of TSB and the culture was incubated until it reached full growth at 37 °C (culture 1). An aliquot of 10 μL was transferred from culture 1 to 10 mL TSB tubes containing 100 mg/L of OXA and the culture (culture 2) was incubated at 37 °C until it reached the maximum OD (ca. 16 h). Employing this procedure, MRSA was serially grown in TSB containing as much as 6400 mg/L OXA.

MIC determination for OXA and ERY was conducted at the beginning of the series and after each stepwise exposure to increasing concentrations of OXA. Determination of susceptibility to kanamycin (KAN), ciprofloxacin (CIP) and amikacin (AMC) was conducted. Kirby–Bauer susceptibility was similarly conducted and the zones of inhibition were measured in millimetres.

2.5. Determination of putative efflux pump activity

The products of each OXA serial culture were subcultured in TSB broth containing 40 mg/L RES and concentrations of ERY ranging from 0.0 mg/L to that of the ERY MIC for each respective serial culture.

2.6. Assessment of efflux pump activity

Assessment of efflux pump activity was conducted by the EB/agar method [7]. This method allows an assessment of overall efflux pump activity of a bacterium by identifying the minimum concentration of EB that produces fluorescence of the bacterial mass on the surface of the agar at 37 °C and that produced after the EB/agar culture is transferred to 4 °C. Evidence of efflux pump activity is noted when the minimum concentration of EB that produces fluorescence at 37 °C is significantly reduced after transfer to 4 °C. Briefly, the products of each serial culture were swabbed onto duplicate agar containing concentrations of EB ranging from 0.05 mg/L to 2.0 mg/L and the plates were incubated at 37 °C for 16 h. At the end of this incubation, the minimum concentration of EB that produced fluorescence associated with the bacterial mass was recorded and then one set of the plates was returned to 37 °C and the other set was transferred to 4 °C. At the end of 16 h of incubation, both sets were examined for the minimum concentration of EB that produced fluorescence of the colonies present on the surface of the agar.

2.7. Purity of the culture products

Purity of the culture products was assessed at the beginning of the series and at the end of the series at 3200 mg/L OXA by DNA typing [8].

3. Results

Growth of MRSA COL following exposure to stepwise increases of OXA is summarised in Fig. 1. Briefly, it is evident that as the concentration of OXA is increased by two-fold increments, the period of time required to achieve