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Increasing Concentrations of Oxacillin Yield Efflux Pump Mediated MDR Phenotype of MRSA COL

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Abstract: MRSA COL strain that is very susceptible to erythromycin (MIC<0.4) and highly resistant to oxacillin (MIC 400 ug mL^{-1}) can be adapted to concentrations of oxacillin as high as 6400 ug mL^{-1}. During adaptation, it slowly becomes increasingly resistant to erythromycin (MIC>30 ug mL^{-1} after adaptation to 3200 ug mL^{-1} of oxacillin). The efflux pump inhibitor rifampin restores initial susceptibility to erythromycin.

Key words: MRSA COL, adaptation to high concentrations of oxacillin, development of mdr, efflux pumps

INTRODUCTION

Exposure of Escherichia coli to step-wise increase of tetracycline (TET) concentrations increases resistance from 2.0 to over 12 mg L^{-1} of TET as well as many other antibiotics and non-antibiotic agents[1]. This mdr phenotype is accompanied by significant increased activity of genes that code for 9 distinct transporter proteins[1]. If we assume that events at the level of the bacterial cell envelop which 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 an mdr type efflux pump. This possibility has been investigated with Methicillin-Resistant Staphylococcus Aureus (MRSA) COL strain sensitive to erythromycin (MIC<0.4 ug mL^{-1}) and highly to oxacillin (MIC 400 ug mL^{-1}) and its exposure to serial increases of oxacillin well beyond its initial MIC.

MATERIAL AND METHODS

Bacterium employed throughout this study was Staphylococcus aureus COL strain generously provided by Prof. Dr. Hermínia de Lencastre. Trypticase Soy was purchased from Difco (Madrid, Spain) and used for the preparation of broth medium (TSB) and agar medium (TSA). OXA, Erythromycin (ERY) in powder form, Ethidium Bromide (EB) and Reserpine (RES) were purchased from Sigma-Aldrich Química SA (Madrid, Spain).

Determination of MIC of antibiotics against Staphylococcus aureus were conducted by the methods previously described[2].

MRSA COL was initially grown in TSB until it reached ½ its maximum Optical Density (OD) as determined spectrophotometrically at 545 nm. An aliquot of 10 μL was transferred to 10 ml tubes containing 50 ug mL^{-1} of OXA in 10 mL of TSB and the culture incubated until it reached full growth at 37°C (culture 1). An aliquot of 10 μL was transferred from culture 1 to 10 mL^{-1} TSB tubes containing 100 ug mL^{-1} of OXA and the culture (culture 2) incubated at 37°C until it reached full growth. Employing this procedure, MRSA was serially grown in TSB containing as much as 6400 ug mL^{-1} of OXA.

MIC for ERY was conducted for the initial strain and after each step-wise exposure to increasing concentrations of oxacillin.

The products of each OXA serial culture were sub-cultured in TSB broth containing 40 ug mL^{-1} of RES and concentrations of ERY that ranged from 0.0 to that of the ERY MIC for each respective serial culture[3].

Assessment of efflux pump activity was conducted by the EB/agar method[4]. Briefly, the products of each serial culture were swabbed onto duplicate agar containing concentrations of EB that ranged from 0.05 to 2.0 ug mL^{-1} and the plates incubated at 37°C for 18 h, examined for the minimal concentration of EB that

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produced fluorescence of the bacterial mass present on the surface of the agar and photographed with the Eagle Eye (Stratagene, USA).

Purity of the culture products were assessed for each serial culture by DNA typing[5].

RESULTS

Exposure of MRSA COL to step-wise increases of OXA and the amount of time needed for the culture to achieve full growth when transferred to increasing concentrations of the antibiotic are summarized by Table 1. Briefly, it is evident that as the concentration of OXA is increased by two-fold increments the period of time required for achieving maximum growth between passages increases significantly. However, after growth transfer from 3200 to 6400 ug mL^{-1} of Oxa, the organism speeds up its replication. No attempt was made to grow

Table 1: Time needed for full growth of culture after transfer to the next increased two-fold concentration of oxacillin [OXA]

[Oxa]	0	50	100	200	400	800	1600	3200	6400
H	8	8	12	24	30	120	160	300	180

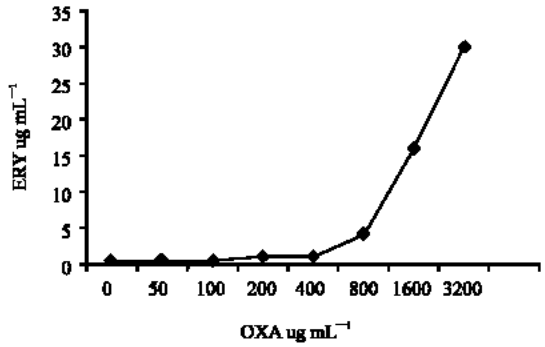

Fig. 1: Resistance of MRSA COL to ERY after prolonged exposure to increasing concentrations of OXA

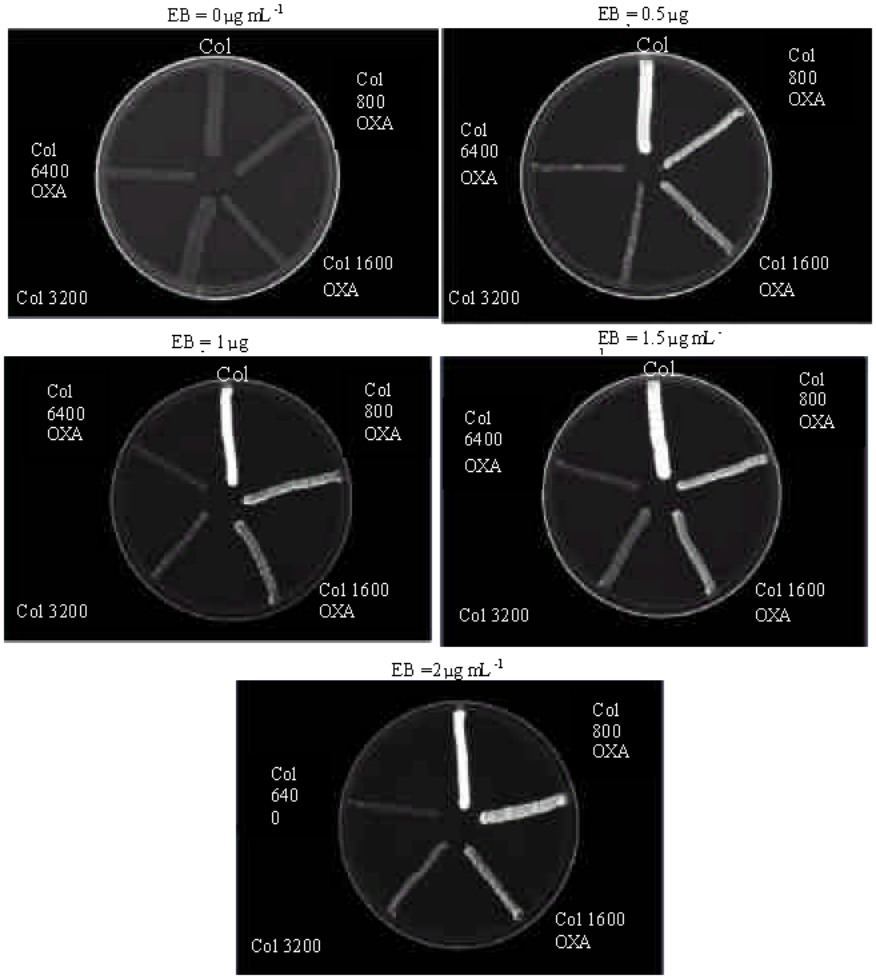

Fig. 2: The effect of exposure of MRSA COL to increasing concentrations of OXA on the minimal concentration of EB that produces fluorescence of colonies

the organism in higher concentrations of the antibiotic. DNA typing of the products at the end of each culture period indicated that the cultures remained free of any contamination (data not shown). MRSA COL is sensitive to ERY (MIC of <0.4 mg L^{-1}). However, at the end of each culture period, as the organism becomes more and more resistant to OXA there is a parallel increase in the MIC of ERY (Fig. 1). The increased resistance to ERY noted after each passage of MRSA COL in medium containing two-fold increases of OXA could be completely eliminated by the addition of 40 mg L^{-1} of RES to the culture (data not shown). The recently developed EB agar method can distinguish degrees of extrusion of EB by assessing the minimal concentration required for the presentation of fluorescence associated with the bacterial mass (4). As extrusion is increased, the minimal concentration of EB needed to produce fluorescence is increased. As evident from Fig. 2 as the organisms becomes adapted to increasing concentrations of OXA the minimal concentration required for the presentation of fluorescence associated with the bacterial mass is increased.

DISCUSSION

The results obtained in the described study demonstrate that when MRSA COL is placed under increasing antibiotic stress, as it adapts to ever-increasing levels of OXA, parallel increases of resistance to ERY take place. Because RES can eliminate the increased resistance to ERY and because the minimal concentration of EB that produces fluorescence of the bacterial mass is also increased in parallel, the mdr phenotype induced by increasing concentrations of OXA is due to an efflux pump. To our knowledge, this is the first demonstration of how an mdr type efflux pump can develop due to continuous exposure to increasing concentrations of an antibiotic to which the organism is chromosomally highly resistant.

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