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Competition between Substrates of the Efflux Pump System of *Salmonella enteritidis*

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Abstract. *Aim: To determine whether tetracycline competes with ethidium bromide (EB), used as substrate of efflux pumps, for extrusion by the efflux pump system of Salmonella enteritidis NCTC 13349 reference strain. Materials and Methods: Evaluation of efflux of EB in competition with tetracycline was conducted with the aid of an automated EB method and by EB agar method. Results: The EB agar method demonstrated that the accumulation of EB by S. enteritidis NCTC 13349 reference strain is reduced by high concentrations of tetracycline and that tetracycline accumulates within the cells. The use of the automated EB method confirmed the EB agar results and demonstrated that accumulation of EB is not affected by tetracycline unless the concentration of the antibiotic exceeds 100 mg/l. Conclusion: These results suggest that tetracycline is preferentially retained and that EB is preferentially extruded by the efflux pump of Sientintidis NCTC 13349.*

Salmonella is the major food-borne pathogen and due to its diverse transient responses to an antibiotic, assumes a multi-drug-resistant (MDR) phenotype. Among these responses is the stepped-up activity of genes that regulate and code for the components of its main efflux pump AcrAB, which may result from either activation of these genes (1), or by invoking either or both of its two-component antibiotic- resistant systems PmrA/B and PhoP/Q (2). Because the AcrAB-TolC efflux pump, as is the case for other Gram-negative bacteria, recognizes and extrudes structurally unrelated antibiotics from at least two classes of antibiotics (3), therapy of salmonellosis is difficult.

The characterization of the intrinsic efflux pump system of *Salmonella enteritidis* NCTC 13349 reference strain is essential for the development of agents that inhibit its main efflux pump AcrAB-TolC. Agents that are capable of this feat could therefore prove useful as adjuvant to common antibiotics to which the efflux pump-mediated MDR organism was initially resistant (3). For the characterization of the intrinsic efflux pump of bacteria, two methods that employ the universal efflux pump substrate ethidium bromide (EB) have been developed (4, 5). Method 1 is the EB agar method that distinguishes and ranks strains of given bacteria that differ with respect to the activity of the efflux pump system over a period of time equivalent to those used in routine bacteriological culture (5); Method 2 employs EB in a broth environment, affords the assessment of the capacity of the efflux system to extrude the EB substrate and evaluates the activity of an agent on the accumulation and efflux of EB over a short period of time (60 minutes) during which no replication takes place due to the absence of nutrients (6). Because our previous study demonstrated that an agent that has been termed an efflux pump inhibitor (EPI) is actually a competitor of the universal substrate EB (6), we have begun a study that evaluates competition between antibiotic substrates of the efflux pump system of the *Salmonella enteritidis* NCTC 13349 reference strain. The study described here, presents, for the first time, evidence obtained by a semi-automated real-time EB method and by the EB agar method that tetracycline competes with EB for extrusion.

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Materials and Methods

Materials. *Salmonella enteritidis* NCTC 13349 reference strain was kindly provided by Seamus Fanning (Centres for Food Safety & Food-borne Zoonomics, UCD Veterinary Sciences Centre, University College Dublin, Belfield, Dublin, Ireland). Muller-Hinton Agar (MHA), Muller-Hinton for preparation of MH broth (MHB), EB, tetracycline and the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), all in powder form were purchased from Sigma, Madrid, Spain. Antibiotic tetracycline containing disks of 30 unit potency were purchased from Difco, Madrid, Spain. Tetracycline E-Test strips purchased from AB Biiodisks, Stockholm, Sweden.

Minimum inhibitory concentrations (MICs) of EB and tetracycline were determined by micro-broth dilution as per Clinical and Laboratory Standards Institute (CLSI) guidelines (7). The effect of EB concentrations on the growth and viability of the bacterium was determined and expressed as colony-forming units (CFUs) previously described (8).

Effect of tetracycline on the accumulation of EB by the EB agar method. The EB agar method employed for evaluation of EB accumulated has been described in detail (8), and most recently in the form of a manual (5). Briefly, a series of MHA plates containing EB from 0.1 to 3.0 mg/l was prepared, swabbed with an overnight culture of the *Salmonella enteritidis* NCTC 13349 reference strain that has been diluted in phosphate-buffered saline (pH 7.4) to match a 0.5 McFarland standard and the plates are incubated at 37˚C for 16 hours. This method determines the maximum concentration of EB that the cell can extrude (no accumulation) and corresponds to no detected fluorescence. Concentrations above this maximum result in increasing fluorescence of the colonies or bacterial lawn, since the capacity of the efflux pump is exceeded (5). After identifying the highest concentration of EB in agar that the cells were able to fully extrude, the plates that contain that concentration of EB were then swabbed with an overnight culture that had been diluted in phosphate-buffered saline (pH 7.4) to match a 0.5 McFarland standard and the plates were incubated at 37˚C for 16 hours. Subsequently, Kirby-Bauer disks containing 0.0, 30 and 70 μg of tetracycline were applied onto the bacterial lawn and the plates returned to the incubator for an additional period of incubation at 37˚C. The plates were then exposed to long wavelength UV by a UVi Tec instrument (UVItec Limited, Cambridge, UK), photographed and the florescence present within distinct areas of the plate determined. If the bacterial lawn emits pink fluorescence when exposed to UV it is because they have not extruded EB, otherwise when the colour emitted is yellow, the cells have accumulated tetracycline and the EB is extruded to the exterior of the cells.

Competition between tetracycline and EB. This was conducted by a semi-automated EB real-time method as previously described in detail for EB and phenyl-arginine-beta-naphthylamide (PAβN) (6). Briefly, incubation of 0.6 OD at 600 nm of the *Salmonella enteritidis* NCTC 13349 reference strain in PBS-0.4% glucose containing different concentrations of EB (1.0, 2.0 and 3.0 mg/l) and tetracycline (0.1 to 1,000 mg/l) were evaluated for the amount of EB accumulated within the cell as previously described (accumulation at 37˚C for up to 40 minutes). The relative fluorescence was provided automatically by a real-time Rotor-Gene™ 3000 thermocycler (Corbett Research, Sydney, Australia) adapted to the EB fluorescence method, namely, temperature of 37˚C, and excitation and emission wavelengths of 530 and 585 nm, respectively (4, 6). The demonstration of efflux of EB was afforded by the use of the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) which binds protons required for the activity of a proton-dependent efflux pump, such as that on which EB efflux is dependent, thereby resulting in significant increases of EB accumulated (6). Accumulation assays were also conducted *via* the above EB method in the absence of glucose in order to limit efflux of EB. This latter assay may afford an understanding of the role of porins in the influx of EB and competition with tetracycline since, in the absence of metabolic energy (glucose), the organism tends to modify its porin activity (9). The use of CCCP in the latter glucose-free assays was conducted in order to reveal any competition between EB and tetracycline.

Results

The MIC of tetracycline against the *Salmonella enteritidis* NCTC 13349 reference strain was 1.25 mg/l by E-Test and confirmed by microbroth dilution; MIC of EB was 128 mg/l. Since the universal substrate EB inhibits the viability of the bacterium at high concentrations (10) and because high concentrations of EB that exceed the capacity of the efflux pump system promote intercalation of EB between the nucleic bases of DNA resulting in a binding constant that prevents ready extrusion (4, 8), it is essential that concentrations of EB employed be substantially low (4, 8). As shown by the EB microbroth dilution method coupled to CFU, a concentration of EB 3.0 mg/l does not appreciably produce accumulation of EB by the NCTC 13349 reference strain nor does it affect the growth of the bacterium (data not shown).

To determine whether tetracycline competes with EB, variations of the EB agar method in combination with increasing concentrations of tetracycline were conducted. As shown in Figure 1, the swabbing of MHA plates containing increasing concentrations of EB followed by incubation for 16 hours at 37˚C indicate that fluorescence produced by the bacterial lawn barely begins with an EB concentration of 3 mg/l in the absence of any tetracycline (plates containing lower and higher concentrations of EB not shown). These results suggest that the bacterial population that makes up the bacterial lawn of the plate is barely able to extrude EB when the concentration of EB of 3 mg/l is reached. When the disk containing no tetracycline was placed on top of the non-fluorescent bacterial lawn and plates then incubated for an additional 24 hours, the bacterial lawn beneath the disk was pink. The presence of increasing concentrations of tetracycline in the disks (30 and 70 μg) promoted only a yellow colour under and around the disk; the greater the amount of tetracycline in the disk, the more intensive and extensive was the yellow colour emitted by the bacteria around the disk. The pink fluorescence of the bacterial lawn increased with distance from the disk, where the concentration of tetracycline decreased. These results suggest that the presence of tetracycline reduces the retention of EB

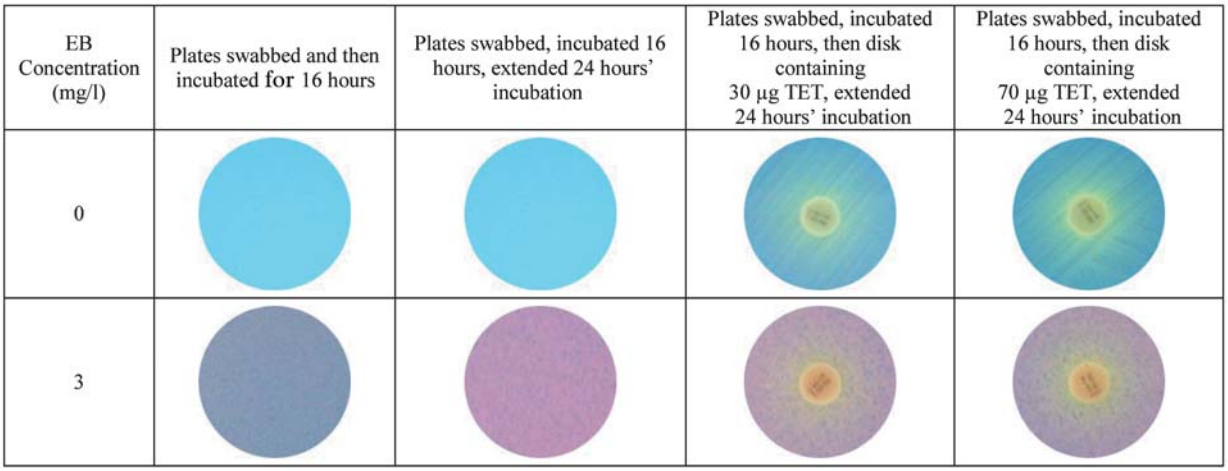

Figure 1. *The effect of tetracycline on the retention of EB by the bacterial lawn. Plates containing increasing concentrations of EB were swabbed with Salmonella enteritidis NCTC 13349 reference strain and incubated for 16 hours at 37˚C. Tetracycline-containing disks of 30 and 70 µg were added and the plates were incubated for an additional 24 hours at 37˚C. A: The plate containing 3 mg/l of EB and no tetracycline disk shows faint pink fluorescence, indicating this is the maximum concentration of EB the cells can extrude. Plates containing lower concentrations of EB were devoid of any fluorescence (not shown). B: The presence of 30 µg of tetracycline promotes formation of a pink-yellow zone around the tetracycline-containing disk of the agar plate containing 3 mg/l of EB. C: At a concentration of tetracycline of 70 µg, the pink-yellow region around the tetracycline-containing disk is replaced by yellow, indicating that EB is preferentially extruded and that tetracycline is preferentially retained.*

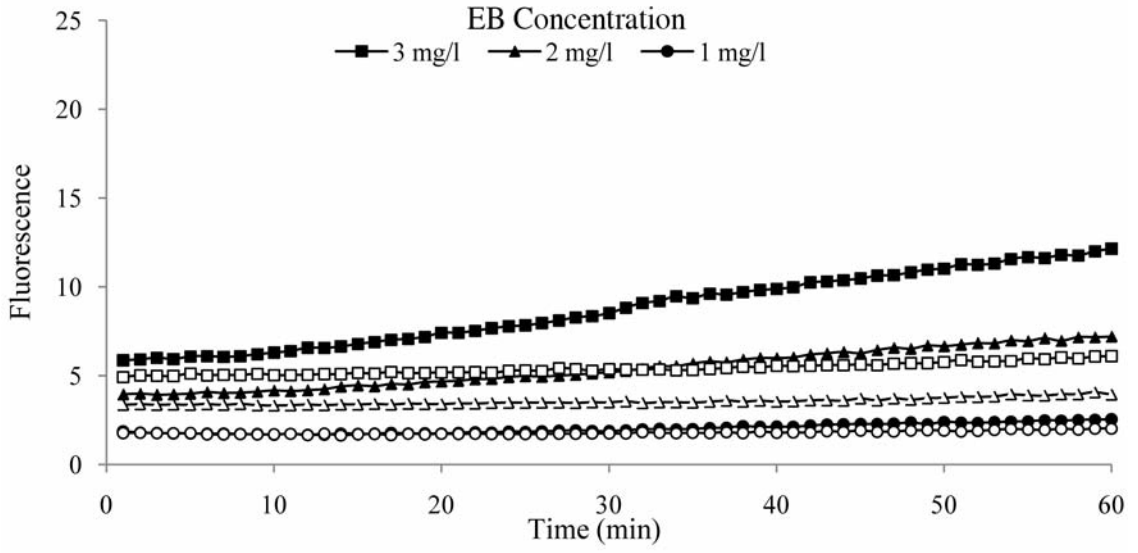

Figure 2. *Effect of glucose on accumulation of EB in Salmonella enteritidis NCTC 13349. Closed symbols, absence of glucose; open symbols, with 0.4% glucose.*

in places where the highest concentration of EB is to be found when no tetracycline is present. Collectively, these results suggest that tetracycline competes with EB for extrusion and that extrusion of EB takes place preferentially. Nevertheless, these interpretations need confirmation and hence the automated EB method that evaluates accumulation/efflux on a real-time basis was conducted.

The use of the automated EB method affords assessment of conditions which affect the accumulation of EB. The amount of EB that accumulates in the cell resulting in an increase of fluorescence during 40 minutes of incubation at 37˚C is affected by pH and by the absence of substrates for metabolic energy production (4, 6, 8). Whereas at pH 5 accumulation of EB has been shown not to be affected by

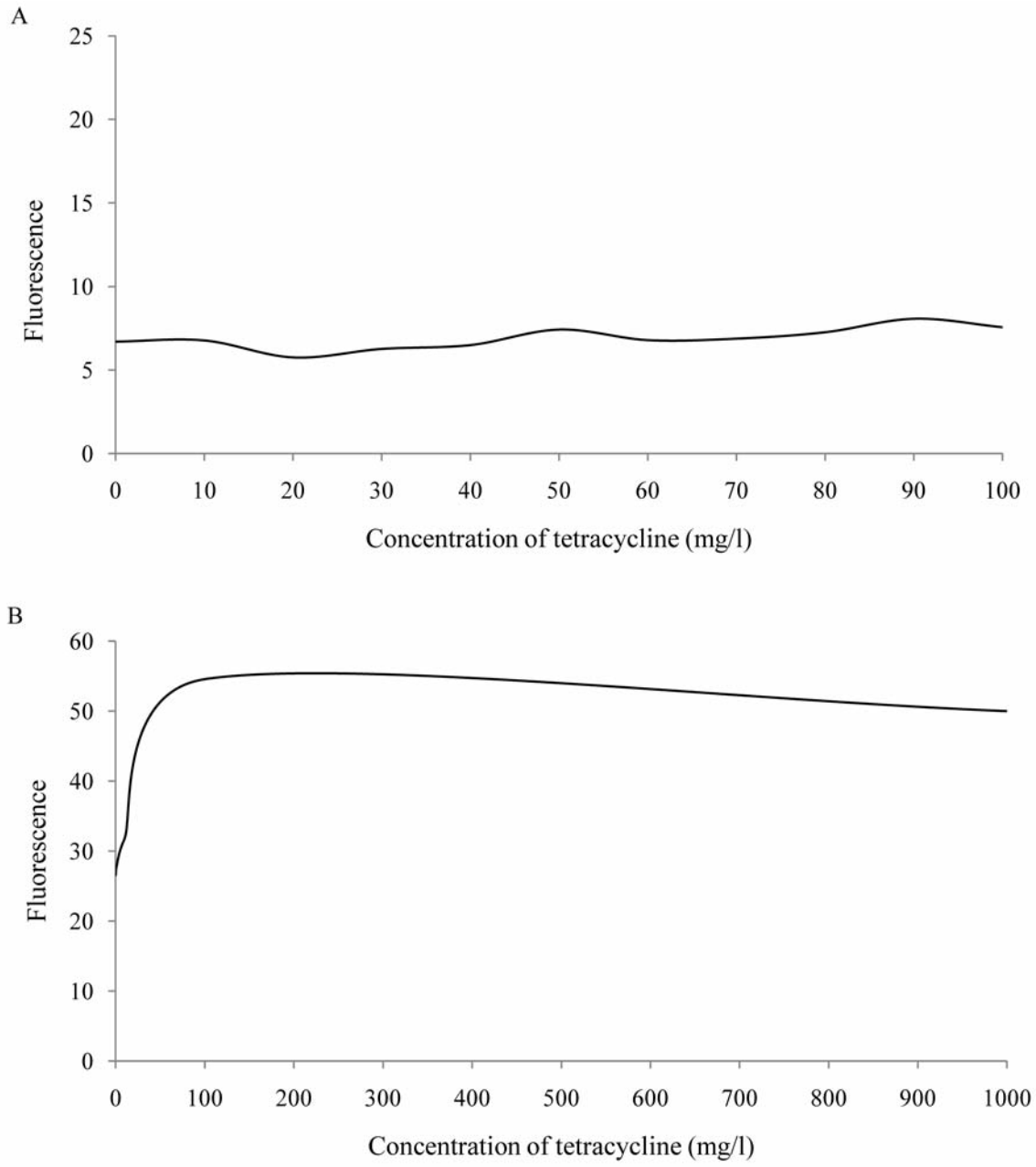

Figure 3. *The effect of the concentration of tetracycline on the accumulation of EB. A: Tetracycline concentrations up to 100 mg/l; B: tetracycline concentrations up to 1000 mg/l.*

the absence of glucose during the 40 minutes of the assay (6, 12, 13), at pH 7.4, the maximum concentration of EB that does not lead to accumulation is 3 mg/l, regardless of glucose availability (Figure 2).

Because at pH 7.4 the degree of accumulation of EB during the assay period of 40 minutes is barely affected by EPIs such as chlorpromazine, PAβN, *etc*. as long as glucose is present in the medium (6), all of the following assays described were conducted at pH 7.4 (PBS). The EB accumulation assay affords an opportunity to study competition between substrates employed by the efflux system of a Gram-negative bacterium at pH 7.4 (6, 11).

As evident from Figure 3A, concentrations of tetracycline as high as 100 mg/l had no effect on the amount of EB accumulated during a period of 25 minutes. At concentrations above 100 mg/l, tetracycline promotes accumulation of EB.

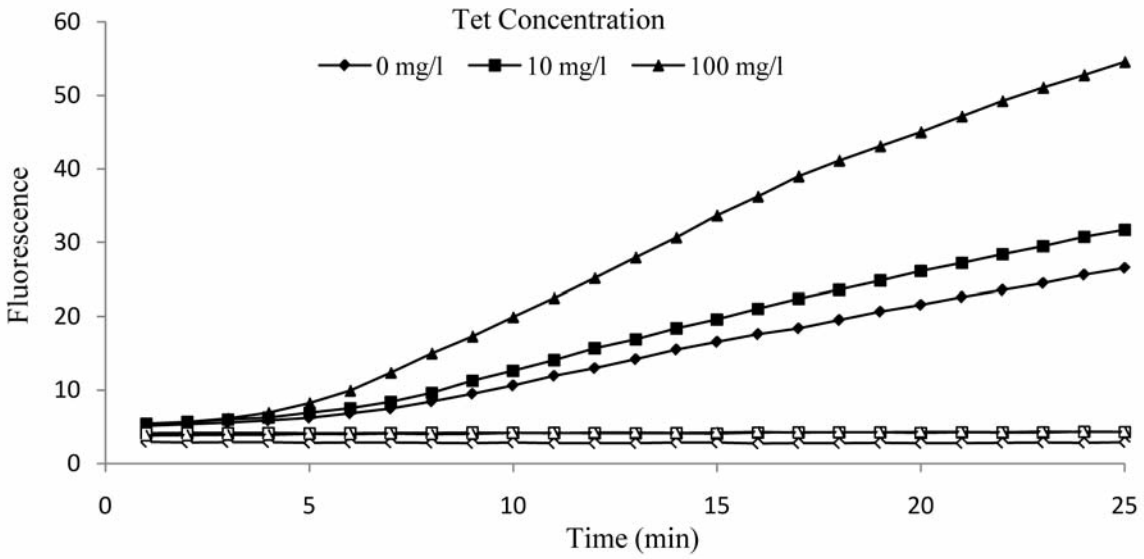

Figure 4. *The effect of increasing concentrations of proton-pump inhibitor CCCP and tetracycline (Tet) on the accumulation of EB in medium containing glucose. The assays containing CCCP (5 mg/l, closed symbols) were conducted at pH 7.4 with glucose at 0.4% and EB at 3 mg/l. Note that in the absence of glucose, there is no significant accumulation of EB. The presence of CCCP increases the amount of EB retained.*

These results coupled to those presented in Figure 1 suggest that tetracycline is preferentially retained while EB is preferentially extruded, and that a concentration of tetracycline in excess of 100 mg/l (Figure 3B) is needed before there is a concomitant increase of EB retained.

The accepted demonstration of a proton-dependent efflux pump system is normally conducted with the addition of the proton ionophore CCCP, which promotes accumulation of EB (6). As evident from Figure 4, 5.0 mg/l of CCCP increased accumulation of EB significantly during the 25 minutes of the assay. The question of whether the addition of tetracycline to the assays containing CCCP has an effect on the accumulation of EB was investigated. As evident from Figure 4, increased accumulation of EB takes place only with concentrations of tetracycline that equal or exceed 100 mg/l. These results suggest that the effect of tetracycline on the accumulation of EB takes place at a site that is distinctly different from that affected by CCCP.

Discussion

Ethidium bromide and tetracycline are known substrates of the AcrAB efflux-TolC pump system of Gram-negative bacteria such as *Escherichia coli* (4) and salmonellae (5). However, to our knowledge, the question of whether these two substrates compete with each other for extrusion by the main efflux pump AcrAB-TolC has not been studied and reported. Gram-negative bacteria begin to accumulate EB when the concentration of EB in the medium exceeds its capacity to extrude the substrate (4, 8, 13). Consequently, when the bacterium is incubated in medium containing the highest concentration of EB that it is capable of extruding, EB does not accumulate within the cell. The addition of an agent that is known to be a substrate of the same efflux pump system that extrudes EB should present a level of competition that is reflected with some change in the accumulation of EB. In our study, the use of two distinct methods demonstrated that when a Gram-negative bacterium, such as the *Salmonella enteritidis* NCTC 13349 reference strain, is challenged with increasing concentrations of EB and tetracycline, accumulation of EB by the bacterial population is reduced only when the concentration of the antibiotic is exceedingly high (at or above 100 mg/l). These results suggest that EB is recognized and extruded by the AcrAB-TolC system preferentially to tetracycline; hence tetracycline accumulates within the cell.

CCCP is a proton ionophore that inhibits the activity of an efflux pump that is dependent upon the proton motive force (3). The addition of tetracycline to CCCP-containing cultures results in no additional accumulation of EB at concentrations of the antibiotic below 100 mg/l, suggesting that the efflux of EB by the proton motive force-dependent system is not affected by the antibiotic. There is a significant increase in accumulated EB, only when the concentration of the antibiotic exceeds 100 mg/l. This degree of accumulation of EB is similar to that produced by the same high concentrations of the antibiotic alone. Therefore, we may tentatively conclude that the efflux of tetracycline and EB can

take place by different efflux pump systems. Nevertheless it remains for further studies to decipher the reality of this tentative conclusion.

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