Characterization of Intrinsic Efflux Activity of *Enterococcus faecalis* ATCC29212 by a Semi-automated Ethidium Bromide Method

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Abstract. *Enterococcus faecalis is recognized as a multidrug-resistant nosocomial pathogen. The phenotypic basis for this is largely uncharacterized. The intrinsic efflux system of the antibiotic-susceptible E. faecalis ATCC29212 strain was studied using a semi-automated method that assesses accumulation and efflux of the universal efflux pump substrate ethidium bromide (EB). The results show that the intrinsic efflux system of this Enterococcus strain is controlled by energy derived from the catabolism of glucose and the proton concentration of the medium. At pH 5, agents that inhibit efflux pumps in Gram-positive organisms and the proton gradient un-coupler CCCP do not increase accumulation nor inhibit efflux of EB. In contrast, at pH 8, where the proton concentration is 1,000-fold lower, these agents increase accumulation and efflux of EB. These results are relevant to infections produced by E. faecalis and subsequent antibiotic therapy with antibiotics to which the organism is known to be intrinsically resistant.*

Abbreviations: EB: ethidium bromide; TZ: thioridazine; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; EPI: efflux pump inhibitor; VRE: vancomycin-resistant *Enterococcus*; MFS: major facilitator superfamily.

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Enterococcus faecalis and *E. faecium* are recognized as nosocomially transmitted pathogens due to the increased use of medically compromising devices such as intravenous drips (IVs), urogenital catheters and intubations (1). These two species represent the third most common isolate recovered from nosocomial infections and account for 10% of all such infections (2). The isolation rate of *E. faecalis* is about 10 times more frequent than that of *E. faecium* (2, 3).

Among vancomycin-resistant enterococci (VRE) isolates, the proportion of *E. faecium* resistant to vancomycin continues to rise in patients hospitalized in the United States (3) *E. faecium* still represents the vast majority of VRE strains. However, given current epidemiological trends, it is reasonable to expect that as these nosocomially acquired infections increase, the development of antibiotic resistance among isolates will follow. As more multidrug-resistant (MDR) strains are reported (4, 5), it is to be expected that therapeutic problems will also increase. The nosocomial route of infection is not the only means by which these pathogens can be disseminated, dairy products (6) have also been implicated as a cause of some pathology.

Overexpressed efflux pumps that extrude a variety of structurally unrelated antibiotics are associated with MDR phenotypes in Gram-negative (7-9) and Gram-positive bacteria (10). In *E. faecalis*, 34 potential multidrug resistance-encoding genes have been identified (11) and two have been characterized: EmeA, a member of the major facilitator superfamily (MFS) and a homolog of NorA (12, 13), and EfrAB, which belongs to the ATP-binding cassette (ABC) superfamily of multidrug efflux transporters (14). Efflux pumps of the MFS family derive their energy from the proton (or sodium) electrochemical

transmembrane gradient, whereas the efflux pumps which belong to the ABC transporters derive their energy from ATP (15). Therefore, it seems that at the very least, two of the efflux pumps of *E. faecalis* derive their energy from two distinct sources.

Until recently, the characterization of efflux activity has been essentially restricted to bacteria that overexpress efflux components. With the aid of a newly developed semi-automated method that characterizes efflux activity on a real-time basis (16), the intrinsic efflux system of the *E. faecalis* under defined physiologically relevant conditions was studied. This paper reports on the activity of these two intrinsic systems in response to biochemical signals.

Materials and Methods

Bacteria. Enterococcus faecalis ATCC29212 strain was used and obtained from the American Type Culture Collection (ATCC).

Materials. Media employed in this study: Mueller-Hinton (MH) in powder form from Oxoid Ltd. (Basingstoke, Hampshire, UK) for the preparation of broth and agar; reserpine, thioridazine (TZ), ethidium bromide (EB), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) purchased from Sigma-Aldrich Química SA (Madrid, Spain); tryptic soy broth (TSB) purchased from Scharlau Chemie S.A. (Barcelona, Spain).

Determination of minimum inhibitory concentrations (MIC). MIC of thioridazine (TZ), ethidium bromide (EB) and reserpine against *Enterococcus faecalis* ATCC29212 was assessed by the broth microdilution method according to CLSI guidelines. Briefly, the strain was cultured overnight at 37°C in MH broth and the inoculum prepared by adjusting the optical density of the culture to the 0.5 McFarland standard. Aliquots of 20 μl were dispensed to each well of a 96-well microplate that contained serial two-fold dilutions of TZ, reserpine and EB. The microplates were incubated at 37°C and the results registered after 16 and 18 hours. The MIC was defined as the lowest concentration of the agent that produced no detectable evidence of growth (turbidity). The MIC for each agent was performed at least three distinct times.

'Real-time' accumulation of EB. This was performed using a previously developed semi-automated method (16). Briefly, the study strain was cultured in TSB until reaching an optical density (OD_{600}) of 0.6 (1×10^8 CFU/ml), following which the tubes (containing 1 ml of bacterial cell suspension) were centrifuged at 13,000 rpm for 3 minutes and the supernatant discarded. The recovered pellet was re-suspended in 1 ml of phosphate-buffered saline (PBS) at pH 7.4 and centrifuged as before. The final washed pellet was then re-suspended in tubes containing PBS buffered to different pH ranging from 5 through 8. The final OD_{600} was adjusted to 0.6.

Aliquots (50 μL) of the bacterial suspension were transferred to 0.2 ml PCR microtubes containing 50 μl of PBS at different pH and concentrations of EB, with or without glucose, and different concentrations of an efflux pump inhibitor (EPI). The final OD_{600} of the bacterial culture was 0.3. The tubes were immediately placed into a Rotor-Gene 3000™ (Corbett Research, Sydney, Australia) thermocycler programmed at 37°C, and for the length of cycle in minutes and total number of cycles according to its contents.
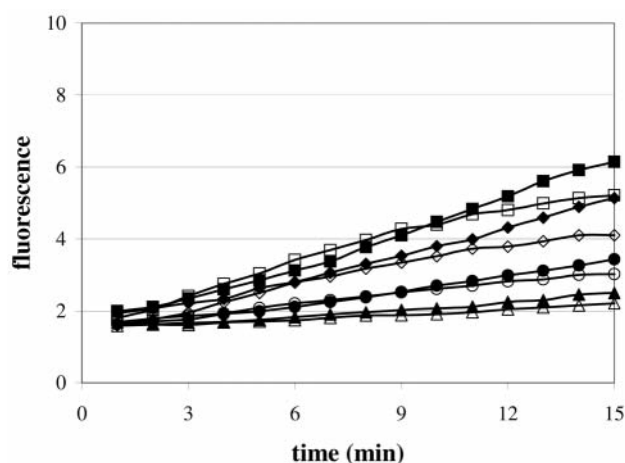

Figure 1. *Accumulation of ethidium bromide (0.5 mg/l) by E. faecalis in medium that is buffered at pH 5, 6, 7 and 8 in the presence and absence of glucose for a period of 15 minutes.* ▲ *pH 5 no glucose,* △ *pH 5 with glucose,* ● *pH 6 no glucose,* ○ *pH 6 with glucose,* ◆ *pH 7 no glucose,* ◇ *pH 7 with glucose,* ■ *pH 8 no glucose,* □ *pH 8 with glucose.*

Assessment of efflux of EB. In order to assess the efflux, sufficient accumulation of EB must first be established. Therefore, conditions which maximize accumulation of EB were employed and accumulation of EB at 37°C allowed for a specific period of time after which the instrument was stopped. Various reagents (glucose, TZ, CCCP) that would allow definition of their effects on efflux were then added in a volume of 10 μl (controls received 10 μl of PBS of matching pH), the instrument was re-started and any change in fluorescence recorded during the remainder of the run (16).

Results

Because an ABC type efflux pump (14) and an MFS type (12, 13) were to be characterized, the accumulation of EB by this strain in the presence and absence of glucose and in PBS solutions at pH ranging from 5 through 8 was initially studied. Alteration in accumulation and efflux of EB is described under well defined conditions as follows.

The effect of EB concentration, pH and viability on the accumulation of EB. If conditions that affect the accumulation of EB are to be studied it is important that the lowest concentration of EB that is barely accumulated be determined. This concentration was found to be 0.5 mg/l of EB (data not shown). Employing this concentration under different conditions of the medium (pH 5 to 8; presence and absence of glucose), the amount of accumulation of EB by *E. faecalis* is minimized by glucose at high pH and by decrease of pH (Figure 1). At pH 5 the absence of glucose has no effect on the amount of EB accumulated. These results suggest that accumulation of EB is reduced when the proton concentration of the medium is high and increases
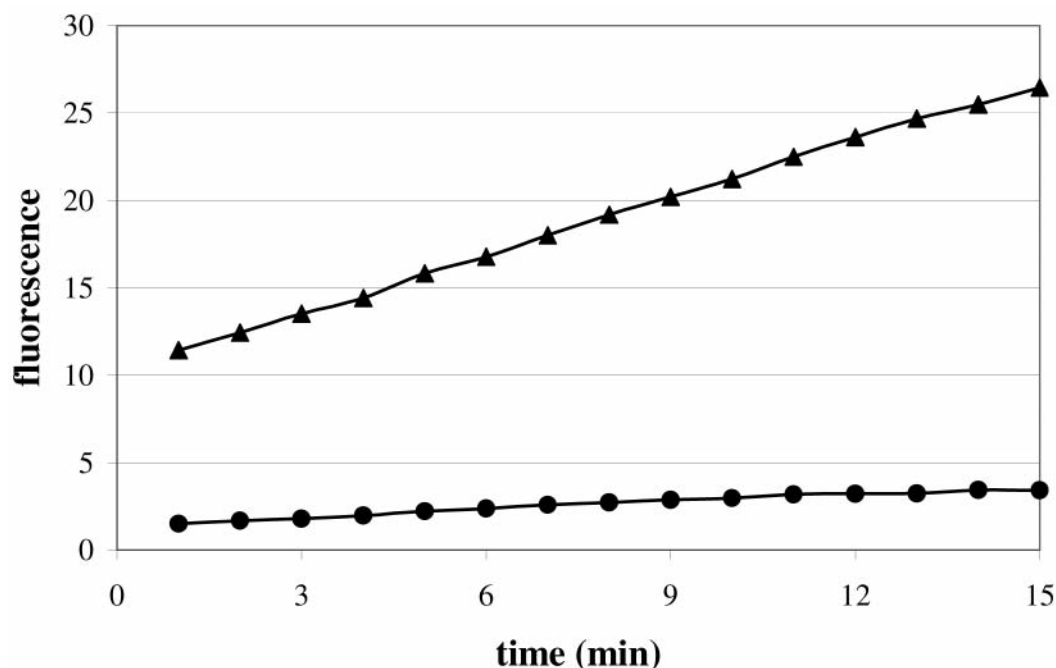
Figure 2. *Accumulation of ethidium bromide (0.5 mg/l) by living and dead cells of E. faecalis at pH 7 in the presence of glucose 0.6% for a period of 15 minutes. Cells were aliquoted evenly: one portion received absolute ethanol to yield 70% ethanol and the other received a blank. The pH of both was adjusted to 7.0. ● Living cells, ▲ dead cells.*

with reduced concentrations of protons as shown for pH 8. This in turn suggests that because glucose minimizes accumulation of EB at pH 8, accumulation at this pH may reflect the activity of an ATP-based efflux system. In contrast, at low pH where the proton motive force is greatest, the accumulation of EB is minimized. These results suggest that the extrusion of EB by *E. faecalis* ATCC29212 takes place *via* pH-dependent pathways.

Cell death. The next condition which impacts on the accumulation of EB is cell death. When the population of bacterial cells is killed following a one hour exposure to 70% ethanol and then transferred to medium containing 0.5 mg/l EB containing 0.6% glucose, as shown by Figure 2, EB is accumulated quickly by dead cells with a total accumulation of EB at the end of 15 minutes that is six-fold greater as compared to the same number of live cells (cells not exposed to ethanol). It should be noted that the EB accumulated does not leak out from the dead cells (16). Furthermore, extraction of DNA demonstrated the presence of EB. EB intercalates between the base pairs of DNA (17) which could be eliminated by incubation of the DNA-EB with DNAse (data not shown).

Effects of EPI reserpine and TZ on accumulation. The EPIs reserpine and TZ have been shown to increase the accumulation of EB by Gram-positive bacteria probably through the inhibition of substrate efflux (19, 20). The

effects of these EPIs were assessed on the accumulation of EB at pH 5 and pH 8 in the presence and absence of glucose. Figure 3 shows that 8 mg/l of TZ promotes accumulation of EB at a pH of 8.0 particularly when glucose is absent from the medium. At the lower pH of 5.0 TZ has no effect on the accumulation of EB regardless of whether glucose is present or absent. The effects of TZ on accumulation of EB in the absence of glucose decrease in magnitude as the pH is lowered (data not shown). Similar results were obtained with 40 mg/l reserpine (data not shown).

The effect of the CCCP on the accumulation of EB at pH 5 and 8. CCCP has been used previously to show that accumulation and efflux of EB is dependent upon a transmembrane proton gradient (22-24). The concentrations of CCCP reported range from 20 to 100 μM (5, 25) and these have been shown to increase the accumulation of EB in both Gram-negative (16) and Gram-positive bacteria (26). It has been observed that CCCP resulted in the killing of bacteria that were exposed to 20 μM (16, 26). Since Figure 2 shows that dead cells accumulate 6 times more EB than live cells, it is difficult to attribute increased accumulation of EB to the collapse of the membrane potential that results from exposure to CCCP. For this reason, the effect of 15 minutes' exposure to increasing concentrations of CCCP on the viability of *E. faecalis* was determined at pH 5 and 8. The results of this viability assay showed that a

concentration as high as 100 μM CCCP does not kill *E. faecalis* at either pH (data not shown), it significantly increases accumulation of EB at pH 8; at pH 5, CCCP has no effect on accumulation of EB (data not shown). These results suggest that the effect of CCCP is most prominent when the proton concentration is low, as would be expected at pH 8, and less prominent with decrease of pH.

Efflux of EB by E. faecalis and the conditions which affect it. Efflux of EB can be demonstrated using the semi-automated method previously described (16, 26). Firstly, to demonstrate efflux, accumulation of EB must be substantial but not extensive to the point where some or most of the EB that enters the cell is intercalated between DNA base pairs (17). EB that intercalates with DNA is not subject to efflux but contributes to the total sum of all accumulated EB. Experimental conditions that allow significant steady-state accumulation of EB by the *E. faecalis* strain over a period of 15 minutes were used (in the absence of glucose and the presence of 0.5 mg/l EB at pH 8 and 5), after which time the instrument was stopped and glucose was then added to replicates and the pH of the media adjusted to 5 and 8. Controls received an equivalent volume of PBS at each pH. The instrument was restarted and fluorescence measurements continued. As the instrument is stopped and then restarted a drop in the fluorescence curve will take place. The extent of the drop is dependent upon the number of minutes the instrument remains in a "stop mode". When the instrument is restarted the component of the curve after the stop and restart describes the degree of efflux over the next 15 minutes. As shown by Figure 4, at pH 8, the addition of glucose after 15 minutes causes a reduction in the amount of fluorescence. At pH 5 the amount of EB accumulated prior to the stop is not affected after the stop, regardless of whether glucose is added.

Similar experiments were conducted to evaluate the effect of TZ on accumulation of EB under varying pH and presence and absence of glucose. Firstly, at pH 8 the addition of TZ in the absence of glucose causes increased accumulation of EB (Figure 5); at pH 5 TZ does not cause accumulation regardless of the absence of glucose (data not shown).

Since TZ is an effective EPI only at high pH, it is proposed as a useful agent for the study of efflux systems that derive their operating energy from the hydrolysis of ATP.

Because CCCP caused an increase in the accumulation of EB at pH 8 in a concentration dependent manner, concentrations of CCCP that ranged from 5 to 100 μM were studied for any direct effect on the efflux of EB at pH 5 and 8. As shown by Figure 6 CCCP decreases efflux only at pH 8 and only with a concentration of 100 μM. At pH 5 CCCP had no effect on efflux of EB. Again, because at pH 5 the contribution of protons to the proton motive force is significantly greater than at pH 8, the demonstration of a CCCP effect on the activity of an efflux pump system may
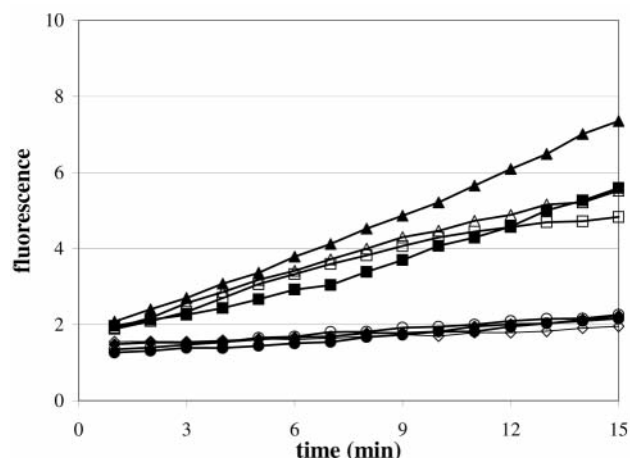

Figure 3. *Accumulation of ethidium bromide 0.5 mg/l by E. faecalis in the presence of TZ (8 mg/l) at pH 8 and 5 in medium containing and lacking glucose 0.6% . ■ pH 8 Control without glucose, □ pH 8 control with glucose, ▲ pH 8 TZ 8 mg/l without glucose, △ pH 8 TZ 8 mg/l with glucose, ◆ pH 5 control without glucose, ◇ pH 5 control with glucose, ● pH 5 TZ 8 mg/l without glucose, ○ pH 5 TZ 8 mg/l with glucose.*

best be evident at pH above 7. Interestingly, all studies employing CCCP for purposes of assessing efflux activity have been conducted with a pH that is close to neutrality.

Discussion

The results obtained in this study suggest that the efflux of EB by *E. faecalis* is primarily controlled by the proton motive force. When the proton motive force is low, the efflux system relies on the hydrolysis of ATP for the provision of protons needed to drive efflux pump systems.

The study of accumulation of EB and its increase due to agents which are considered to be EPIs is invariably conducted by others at pH 7, at room temperature and in media lacking glucose. These conditions fail to mimic the physiological conditions existing in the environment of a bacterial cell. The methodology and real-time evaluation of accumulation and efflux afforded by the semi-automated method provides a somewhat closer representation of physiological conditions at sites of infection that afford a more defined assessment of overall activity of the efflux pump system.

Although the results obtained in this study define the efflux activity of a proton motive force-dependent efflux system possibly supplemented by one that relies on the immediate energy provided by the hydrolysis of ATP, until the current study is reproduced with a mutant of *E. faecalis* whose MFS EmeA efflux pump has been deleted, it is difficult to uncover any synergies that may exist between both efflux systems. Moreover, because as much as 35% of
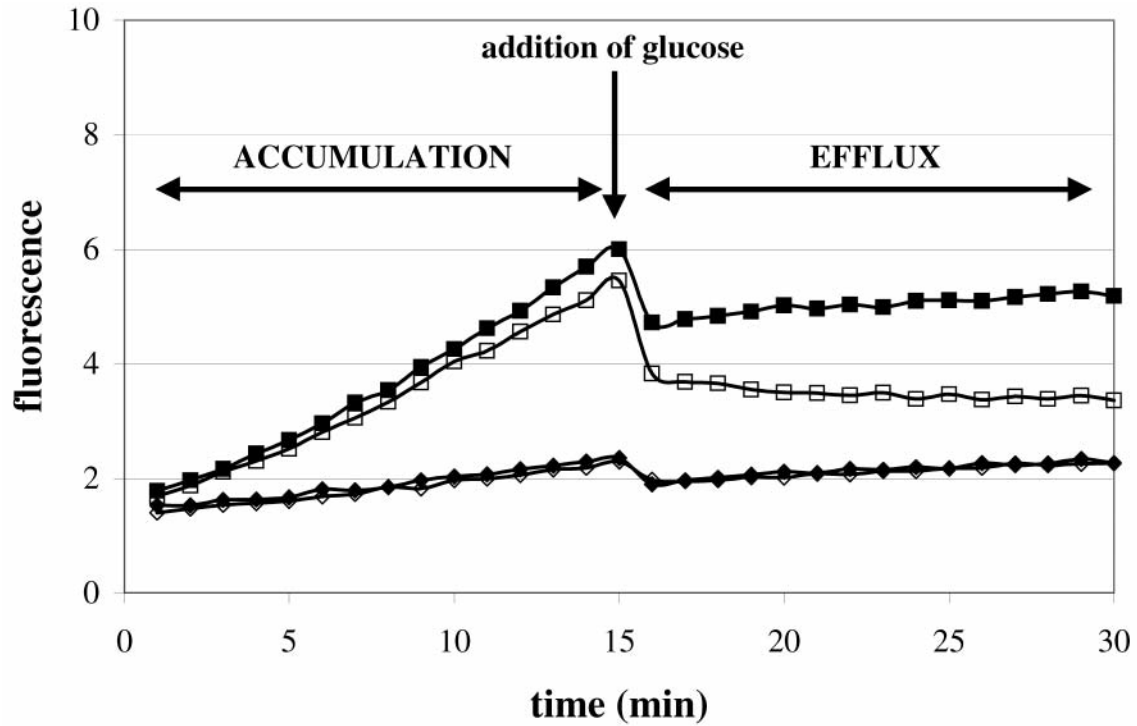
Figure 4. *Accumulation efflux of ethidium bromide (0.5 mg/l) by E. faecalis in the presence and absence of glucose 0.6% at pH 8 and pH 5. Glucose was added after the accumulation period (15 minutes) to the samples.* ■ *pH 8 without glucose,* □ *pH 8 with glucose,* ◆ *pH 5 without glucose,* ◇ *pH 5 with glucose.*
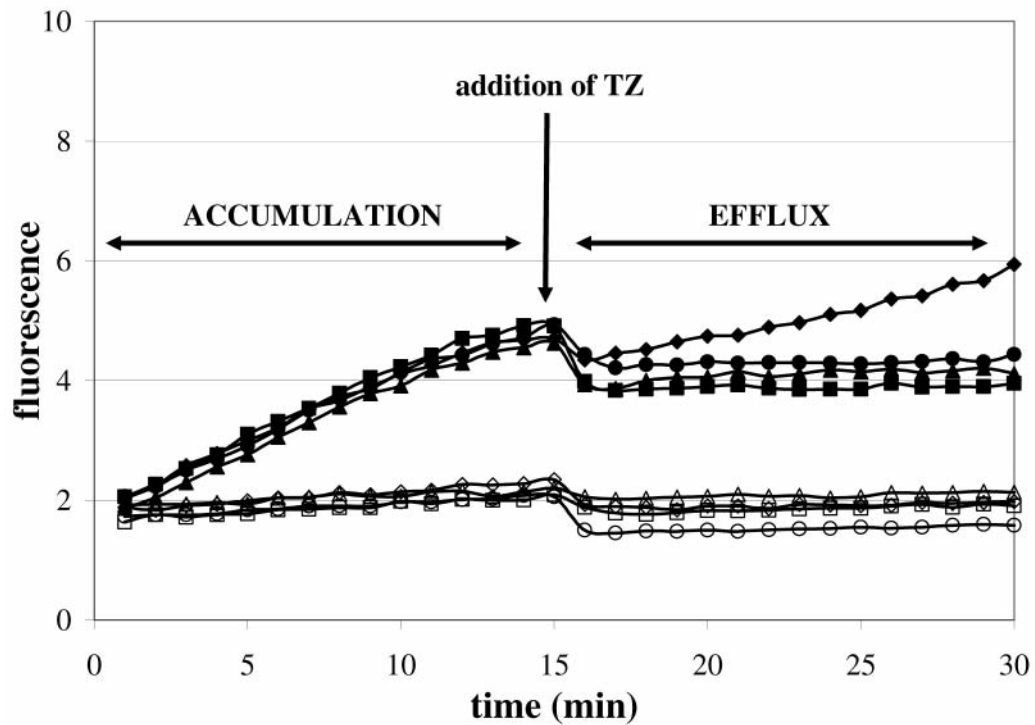

Figure 5. *Accumulation efflux of ethidium bromide (0.5 mg/l) by E. faecalis in the presence of glucose 0.6% after adding TZ at different concentrations (8, 12 and 16 mg/l) at pH 8 and pH 5. TZ was added after the accumulation period (15 minutes) to the samples.* ▲ *pH 8 Control,* ■ *pH 8 TZ 8 mg/l,* ● *pH 8 TZ 12 mg/l,* ◆ *pH 8 TZ 16 mg/l.* △ *pH 5 Control,* □ *pH 5 TZ 8 mg/l,* ○ *pH 5 TZ 12 mg/l,* ◇ *pH 5 TZ 16 mg/l.*
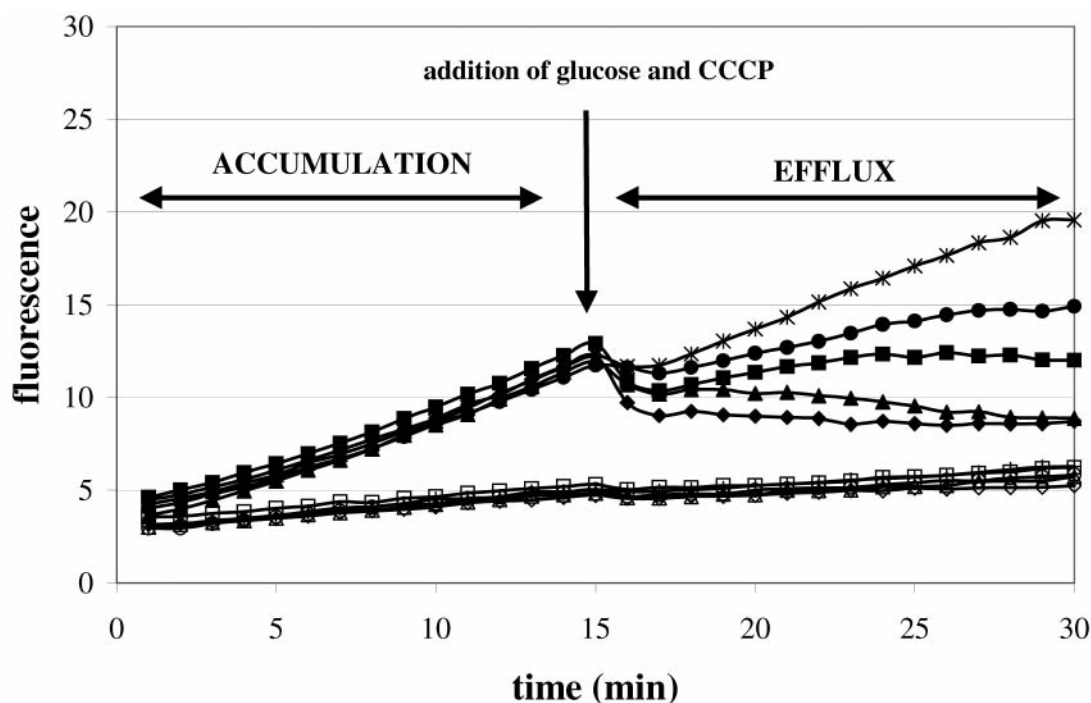
Figure 6. *Accumulation efflux of ethidium bromide (0.5 mg/l) by E. faecalis with CCCP (5, 10, 20 and 100 μM) and glucose 0.6% at pH8 and pH5. CCCP and glucose were added after the accumulation period (15 minutes) to the samples.* ◆ *pH 8 Control,* ▲ *pH 8 CCCP 5 μM,* ■ *pH 8 CCCP 20 μM,* ● *pH 8 CCCP 50 μM, *pH 8 CCCP 100 μM,* ◇ *pH 5 control,* △ *pH 5 CCCP 5 μM,* □ *pH 5 CCCP 20 μM,* ○ *pH 5 CCCP 50 μM, + pH 5 CCCP 100 μM.*

the bacterial genome is devoted to the coding of transporters (18, 28), other efflux pumps yet to be identified may similarly contribute to the overall effects observed in this study.

An EPI should by definition reduce resistance and make a bacterium increasingly more sensitive to a given antibiotic or even reverse the MDR phenotype (18, 21, 25, 29). This latter definition is based upon prolonged contact with the EPI, usually at least 16 hours' duration. The inability to observe any EPI effect on accumulation and efflux of EB at pH 5 may be due to the short amount of time the organism was in contact with the EPI (maximum 30 minutes). However, cultures that define the MIC of CCCP, TZ and reserpine were conducted at pH 5 and 8. The MICs of these EPIs are significantly lower at pH 8 than at pH 5 (data not shown). Therefore, in all probability, at pH 5 an EPI is ineffective regardless of the amount of time the organism is exposed to it. This observation may have some clinical relevance inasmuch as bacteria that produce infections in the stomach and urinary tract are more resistant to given antibiotics than when the infection is elsewhere (30, 31). Based upon the results obtained in this study, it is deemed probable that the intrinsic resistance of the bacterium in organs that have low pH is due to the intrinsic efflux system of the bacterium that extrudes the antibiotic before it reaches its intended target.

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