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Deletion of the Multiple-Drug Efflux Pump AcrAB in *Escherichia coli* Prolongs the Postantibiotic Effect

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The mechanism of the postantibiotic effect (PAE) was examined in *Escherichia coli*. Drugs exhibited longer-lasting PAEs in an *acrAB* mutant, suggesting that intracellular drug concentrations influence the duration of the PAE. With specific assays for tetracycline and erythromycin, a direct link between intracellular persistence of antibiotics and maintenance of the PAE was established.

The mechanism of the postantibiotic effect (PAE), which is the temporary suppression of bacterial growth following short exposure to an antimicrobial agent (3), is poorly understood.

Saturable, dose-dependent increases in PAE duration have been observed in several organisms after exposure to different agents (1, 5), suggesting that the PAE depends on temporary inactivation of cellular drug targets. Resumption of growth after the PAE period could therefore reflect the time taken for antimicrobial agents to dissociate from their targets and be lost from the cell, releasing sufficient target molecules for growth to resume. Alternatively, resynthesis of the drug target may be required for recovery after the antibiotic has been released from the cell (2, 11). In either case, maintenance of the PAE is predicted to depend on antibiotic persistence within the cell and accordingly the duration of the PAE is expected to be related to the intracellular drug concentration. Nevertheless, the effects of intracellular concentrations of antimicrobial agents on the PAE have not been extensively examined.

We investigated the duration of the PAE induced by various drugs in an *Escherichia coli* mutant deficient in the multiple-drug efflux pump AcrAB. The drugs produced longer-lasting PAEs in the *acrAB* mutant, consistent with prolonged target interactions mediated by maintenance of higher intracellular drug concentrations in the efflux-deficient strain. Furthermore, by using specific assays for tetracycline (TET) and erythromycin (ERM) we established a direct link between the intracellular persistence of these antibiotics and maintenance of the PAE in *E. coli*.

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Most antimicrobial agents, apart from β-lactams, induce PAEs in *E. coli* (3). For PAE studies, we therefore investigated agents other than β-lactam antibiotics, focusing on chloramphenicol (CAM), ERM, fusidic acid (FUS), mupirocin (MUP), nitrofurantoin (NIT), novobiocin (NOV), puromycin (PUR),

rifampin (RIF), and TET, which are known to induce PAEs in *E. coli* (3, 9). With the exception of NOV, these agents have also been reported to induce PAEs in species other than *E. coli* (3, 9). Therefore, apart from NOV, for which there are apparently no PAE data other than those for *E. coli*, the majority of the agents studied here induce PAEs in species other than *E. coli*. Consequently, with the exception of NOV, it can be concluded that the PAE-inducing activity of the other eight drugs studied here (CAM, ERM, FUS, MUP, NIT, PUR, RIF, and TET) is not species specific.

We sought to establish whether enhanced retention of antimicrobial agents in an *acrAB* mutant of *E. coli* would lengthen the PAE. We focused on CAM, ERM, FUS, NOV, PUR, RIF, and TET, whose activity is known to be influenced by expression of the AcrAB multidrug efflux pump (10). It should be noted that some of these agents might also undergo efflux by pumps other than AcrAB, although no biochemical data on the involvement of additional pumps have been obtained (10). In addition, we examined MUP and NIT as further agents that might be substrates for AcrAB. The susceptibility of *E. coli* K-12 strain SM1411 (*acrAB*) (7) to these agents was determined and compared with that of isogenic parent strain 1411 (Table 1). MICs were determined by broth microdilution in Mueller-Hinton broth with an inoculum of 10^4 cell/ml in a final volume of 70 μl.

Inactivation of *acrAB* caused increased susceptibility to all of the antimicrobial agents that we examined (Table 1). The increases in susceptibility ranged from 1.6-fold with RIF to 128-fold with FUS. The results are therefore consistent with the removal of these antimicrobial agents by AcrAB. The data for MUP and NIT are novel, and the observations with the other seven agents agree with those of Sulavik et al. (10).

PAEs were determined in Mueller-Hinton broth by a microplate method after exposure of bacteria to antimicrobial agents for 60 min, followed by centrifugation and washing of organisms to remove extracellular drug (9). The method used here for determining the PAE differs from the classical agar-based method (3) since it is based on measuring culture optical density at 600 nm (OD_{600}) in a microplate reader (9). Bacteria were exposed to antimicrobial agents at concentrations equal to five times the respective MICs for strain 1411. PAE durations for all of the drugs were significantly longer (to within 95% confidence limits, as determined by Student's *t* test) in

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TABLE 1. Effect of inactivation of *acrAB* on susceptibility to
antimicrobial agents and the duration of the PAE

Antimicrobial agent	MIC (μg/ml)		PAE duration (h)[a]	
	1411 (*acrAB*[+])	SM1411 (*acrAB*)	1411 (*acrAB*[+])	SM1411 (*acrAB*)
CAM	4	0.25	1.23 ± 0.29	2.23 ± 0.22
ERM	32	2	0.68 ± 0.16	2.71 ± 0.2
FUS	256	2	0.41 ± 0.04	4.40 ± 0.2
MUP	32	1	0.59 ± 1.03	1.10 ± 0.19
NIT	4	1	1.21 ± 0.34	5.50 ± 0.44
NOV	64	1	0.84 ± 0.24	3.06 ± 0.74
PUR	64	4	1.35 ± 0.4	3.40 ± 0.33
RIF	4	2.5	4.06 ± 0.72	7.41 ± 0.44
TET	4	1	4.45 ± 0.26	>40

[a] Determined by the microplate method. Each PAE value is the mean of three determinations ± the standard deviation.

SM1411 than in 1411 (Table 1). Increases varied from 2-fold (CAM) to 10-fold (FUS and TET).

The data presented above suggested a relationship between the intracellular drug concentration and the duration of the PAE since PAEs lasted longer in the *acrAB* mutant, where the rate of drug efflux is decreased. To establish a direct link between a persistent intracellular drug and the PAE, we determined the intracellular concentrations (4, 8) of two model antibiotics, TET and ERM, throughout the PAE and examined whether recovery from the PAE coincided with loss of drug from the cell. The method used was based on the microplate method that had been used to determine the PAE values listed in Table 1 but scaled up to the milliliter level to permit sufficient sample sizes for determination of intracellular antibiotic pools. Consequently, the PAE values obtained in these experiments (Fig. 1 and 2) are not strictly comparable to those listed in Table 1.

The PAE induced by TET in *E. coli* 1411 and the intracellular TET concentration at hourly intervals are shown in Fig. 1a. $T_{0 h}$ represents the point immediately after washing to remove extracellular drug from the medium. In this experiment, the period of growth inhibition lasted around 2 h before the cells began to recover. The PAE duration, defined in accordance with reference 6 and Stubbings et al. (9), was 3.2 h. The intracellular concentration of TET at $T_{0 h}$ was 0.1 mmol/OD_{600} unit. TET remained at this level throughout the 2-h period of growth inhibition and then decreased to zero over the next 2 h. The PAE induced by TET in SM1411 and the intracellular TET concentrations at time points throughout this period are shown in Fig. 1b. Exposure to a concentration equal to five times the parental MIC for 1 h induced a PAE that was more than 10-fold longer than in the parent strain, 1411. The intracellular TET concentration of SM1411 remained between 0.3 and 0.4 mmol/OD_{600} unit throughout the PAE and then decreased to zero when normal growth resumed after 50 h. The reason that growth resumed quite rapidly at 50 h in the *acrAB* mutant is unclear but may represent induction of systems other than AcrAB that are capable of removing TET from the cell.

The PAE induced in *E. coli* 1411 by a concentration of ERM equal to five times the MIC and the intracellular ERM concentration measured at 20-min intervals during the PAE are shown in Fig. 2a. Growth resumed almost immediately follow-
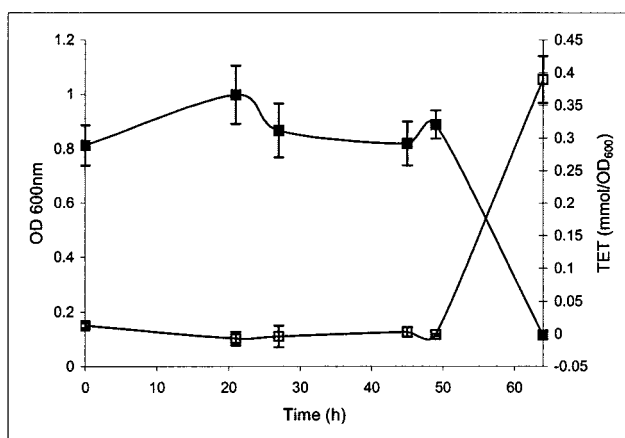
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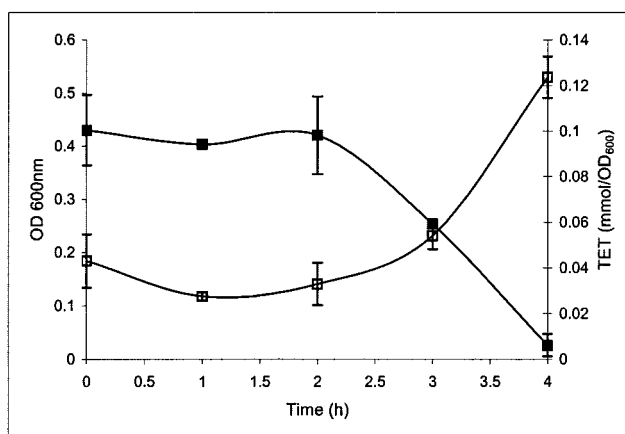
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FIG. 1. Intracellular TET concentrations in *E. coli* 1411 (a) and SM1411 (b) at intervals during the PAE induced by a concentration of TET equal to five times the MIC for the parental strain. Intracellular TET concentrations (■) are expressed as millimoles per OD_{600} unit, and the measured OD_{600} (□) of the culture is shown. Error bars show standard deviations of three replicate values.

ing removal of the drug from the medium, exhibiting a PAE of 0.3 h in this experiment. This correlated with the complete removal of intracellular ERM at time zero. Indeed, ERM was only detectable in strain 1411 when measurements were taken midway through the ERM exposure period, during which drug levels of approximately 0.35 mmol ERM/OD_{600} unit were recorded (data not shown). Figure 2b describes an experiment in which ERM induced a PAE lasting 1.8 h in SM1411. The intracellular ERM concentration was 0.28 mmol/OD_{600} unit at $T_{0 h}$. It then decreased to zero over the following 3 h. The ERM concentration fell to 50% of its original value by $T_{2 h}$, the time at which growth resumed.

In this paper we have reported that longer-lasting PAEs were observed for drugs in SM1411 than in 1411. This suggests a relationship between the intracellular drug concentration and the duration of the PAE, since the longer-lasting PAEs in the *acrAB* mutant are consistent with decreased drug efflux in this strain. Intracellular concentrations of two representative
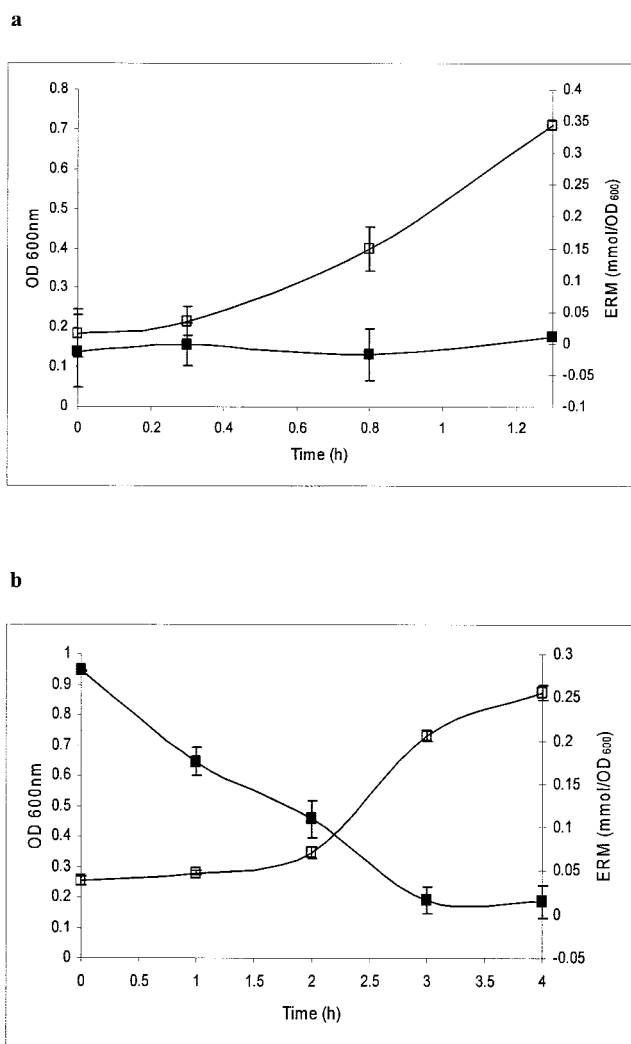
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FIG. 2. Intracellular ERM concentration and bacterial growth at intervals during the PAE. PAEs were induced in *E. coli* 1411 (a) and SM1411 (b) by a concentration of ERM equal to five times the MIC for the parental strain. Intracellular ERM concentrations (■) are expressed as millimoles per OD_{600} unit, and the measured OD_{600} (□) of the culture is shown. Error bars show standard deviations of three replicate values.

antibiotics, TET and ERM, were determined directly during the PAE and recovery periods after exposure to these drugs. Recovery from the PAE coincided with loss of the drugs from the cell, and the intracellular concentrations of the antibiotics remained high throughout the longer-lasting PAEs observed in the *acrAB* mutant.

Nevertheless, differences in behavior were observed between TET- and ERM-inhibited bacteria. In contrast to those of TET, ERM concentrations were not maintained for long periods in either 1411 or SM1411 (Fig. 2), suggesting that a pump additional to AcrAB may be involved in the efflux of ERM. Furthermore, by comparing the data in Fig. 1a and 2a it appears that the AcrAB pump in strain 1411 is immediately effective for the removal of ERM, but not TET, where at least 2 h is required to detect loss of TET from the cell. This could indicate that TET is more firmly associated with the ribosome than ERM such that ERM, by dissociating from the ribosome more rapidly than TET, becomes available for efflux by AcrAB sooner than TET. Alternatively, the AcrAB pump may simply remove ERM from the cell more efficiently than TET.

Despite differences in the responses to TET and ERM, we conclude that the duration of the PAE in *E. coli* is related to the length of time that drug molecules remain within the cell. Our data are in agreement with the only other study that has examined intracellular antibiotic levels during the PAE. Thus, Champney and Tober (2) found that the presence of ERM, bound to the ribosome, was the limiting factor for recovery of *Staphylococcus aureus* from the ERM-induced PAE.

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