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Different Patterns of Bacterial DNA Synthesis during Postantibiotic Effect

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Studies on bacterial metabolism during the postantibiotic effect (PAE) period are limited but might provide insight into the nature of the PAE. We evaluated the rate of DNA synthesis in bacteria during the PAE period after a 1-h exposure of organisms in the logarithmic growth phase to various antibiotics. *Staphylococcus aureus* ATCC 25923 was exposed to vancomycin, dicloxacillin, rifampin, and ciprofloxacin; *Escherichia coli* ATCC 25922 was exposed to gentamicin, tobramycin, rifampin, imipenem, and ciprofloxacin; and *Pseudomonas aeruginosa* ATCC 25783 was exposed to imipenem, tobramycin, and ciprofloxacin. DNA synthesis was determined by measuring the rate of [^{3}H]thymidine incorporation in *S. aureus* and *E. coli* and [^{3}H]adenine incorporation in *P. aeruginosa*. DNA synthesis in *S. aureus* was suppressed during the PAE phase with vancomycin, dicloxacillin, and rifampin, it was suppressed in *E. coli* with rifampin, and it was suppressed in *P. aeruginosa* after exposure to tobramycin. Conversely, DNA synthesis was relatively enhanced in the gram-negative bacilli after exposure to imipenem and in all three species after exposure to ciprofloxacin. However, DNA synthesis in *E. coli* was only minimally affected after exposure to tobramycin and gentamicin. The differences in DNA synthesis observed after exposure to various antimicrobial agents suggest multiple mechanisms for the PAE.

The postantibiotic effect (PAE), defined as the presence of persistent suppression of bacterial growth after a short-term exposure of bacteria to antimicrobial agents, has been well documented both in vitro and in vivo (5, 7, 25). The presence and duration of the PAE, however, differ significantly for various microorganism-antibiotic combinations. Furthermore, the concentration and duration of exposure of the antibiotic, the combinations of antibiotics used, the size of the bacterial inoculum, and the type and pH of the medium all influence the duration or presence of the PAE (5, 7, 16, 17). The clinical significance of the PAE pertains primarily to the impact that it may have on antimicrobial dosing regimens in clinical practice (12, 19, 30).

Only a limited number of studies have addressed bacterial metabolic events during the PAE period (1, 13–15, 20, 27). However, such investigations might add insight into the possible mechanisms underlying this temporary inhibition of bacterial multiplication. The main purpose of our study was to investigate and compare DNA synthesis in bacteria during and after PAEs induced by several different antibiotics in order to gain a better understanding of metabolic activity during the PAE period.

MATERIALS AND METHODS

Organisms and media. The organisms used in the study were *Pseudomonas aeruginosa* ATCC 25783, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923 (American Type Culture Collection, Rockville, Md.). Mueller-Hinton broth (MHB; Difco Laboratories, Detroit, Mich.) served as a culture medium. The broth was supplemented with 50 mg of Ca^{2+} as calcium chloride and 20 mg of Mg^{2+} as magnesium sulfate per 1,000 ml (34) while performing experiments involving *P. aeruginosa.*

Antibiotics. Rifampin was supplied by Ciba-Geigy (Basel, Switzerland), ciprofloxacin was supplied by Bayer AG (Leverkusen, Germany), gentamicin was supplied by Roussel Laboratories Ltd. (Uxbridge, United Kingdom), imipenem was supplied by Merck Sharp & Dohme International (Rahway, N.J.), dicloxacillin was supplied by Bristol-Myers Squibb Pharmaceuticals Ltd., (Merseyside, United Kingdom), and tobramycin and vancomycin were supplied by Eli Lilly & Co. (Indianapolis, Ind.). Stock solutions were prepared in sterile saline and were stored at −20°C until use, except that imipenem and rifampin were dissolved and diluted in phosphate-buffered saline and methanol, respectively, on the day of the experiment. MICs were determined by a standard microtiter dilution method (26).

Inoculum and organism-antibiotic combinations. Before each experiment three to four colonies of the test organism were transferred to 5 ml of MHB, serially diluted, and grown overnight (8 to 10 h) at 35.5°C. Subsequently, the culture was adjusted to a concentration of ~10^7 CFU/ml by using a 0.5 McFarland standard; the organisms were in the logarithmic growth phase. The logarithmic growth phase was confirmed by determining the viable counts of unexposed control organisms while the test organisms were being exposed to antimicrobial agents. DNA synthesis was studied in each organism after 1 h of exposure to the following antibiotics (concentrations in multiples of the MIC): *S. aureus* after exposure to dicloxacillin (2× and 4×), vancomycin (4×), ciprofloxacin (1×, 2×, and 4×), and rifampin (4×); *E. coli* after exposure to imipenem (1× and 2×), gentamicin (2× and 4×), tobramycin (1×), rifampin (4× MIC), and ciprofloxacin (1× and 2× MIC); and *P. aeruginosa* after exposure to imipenem (4× and 8×), tobramycin (2×), and ciprofloxacin (1×, 2×, and 4×).

Chemicals. [6-^{3}H]thymidine and [2-^{3}H]adenine (specific activities, 5.0 and 25.0 Ci/mmol, respectively) were purchased from Amersham International (Amersham, Buckinghamshire, United Kingdom). Trichloroacetic acid (TCA) and NaOH were obtained from Merck (Darmstadt, Germany), bovine serum albumin (BSA) was obtained from Sigma, and EDTA was obtained from BDH Chemicals (Poole, England). Toluene with 5 g of POPOP [2,2′-*p*-phenylene-bis-(phenyloxazole)] and 0.12 g of PPO (2,5-diphenyloxazole) (Merck) per liter served as scintillation fluid.

Drug removal. After 1 h of exposure to antibiotics, the antibiotic-exposed and the unexposed control cultures were filtered through 0.45-μm-pore-size filters (Millipore HA; Millipore Corporation, Bedford, Mass.). The filters were washed with 100 ml of sterile 0.9% NaCl and then 10 ml of MHB. Subsequently, the filters were inoculated in prewarmed MHB, shaken vigorously, and removed.

PAE. The duration of the PAE was calculated by the equation $PAE = T - C$, where C is the time required by control organisms to grow 1 log unit, whereas T is the time necessary for antibiotic-treated bacteria to increase by 1 log unit after drug removal (7).

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DNA synthesis. A modified method of Engle et al. (10) was used to measure DNA synthesis. After the drug removal procedure, 1 ml of each of the *E. coli* and *S. aureus* cultures was pulsed with 10 μCi of [6-^{3}H]thymidine for exactly 10 min. Then, the bacteria were lysed with ice-cold TCA (final concentration, 10% [wt/vol]). The interval between pulsations was ~20 min for the first 1.5 h, after which the interval was increased to 1.5 h.

In initial studies *P. aeruginosa* failed to incorporate the [^{3}H]thymidine (data not shown), consistent with the observations of Pruul et al. (31). Instead, 1 ml of the *P. aeruginosa* cultures was pulsed at the same intervals with 10 μCi of [2-^{3}H]adenine for 10 min. Then, NaOH and EDTA were added to terminate the reaction (final concentrations, 0.3 N and 0.1%, respectively). The tubes were incubated for 8 to 10 h at 37°C to hydrolyze the RNA (2). Then, 40 μg of BSA was added and the DNA was precipitated with TCA (final concentration, 10% [wt/vol]).

The lysed bacteria were transferred to tissue culture microtiter plates, with 200 μl in each well and at each time point in triplicate or quadruplicate. Then, the suspension was harvested with a cell harvester (Titertek, Skatron, Norway) on glass filters and each well was carefully washed with 10 ml of sterile water, which was also passed through the filters. The filters were air dried and the radioactivity was determined with a 1214 Rackbeta liquid scintillation counter (LKB, Wallac, Turku, Finland). The counts per minute were used as an indicator of DNA synthesis at the various time points. Correction was made for background radioactivity.

Viable counts were estimated at 90-min intervals by serial dilution in ice-cold 0.9% NaCl and plating on Mueller-Hinton agar. Since the bacterial counts remain relatively constant early after drug removal, the numbers of CFU were calculated for the various time points (10, 30, 50, and 70 min) by using 0 and 90 min as reference time points. Previously, we demonstrated that viable counts at 50 min corresponded well to the calculated number (data not shown). Most experiments were repeated two to four times on different days.

Analysis of data. The rate of DNA synthesis per organism was calculated and expressed in terms of counts per minute per CFU per minute (4). The correlation between counts per minute and viable counts in untreated control organisms for the first 90 min of growth for *E. coli*, the first 180 min of growth for *S. aureus*, and the first 270 min of growth for *P. aeruginosa* was determined by least-squares linear regression.

The datum points from the triplicate or quadruplicate experiments were averaged, and the final data were expressed as means ± standard deviations (SDs) of the averages from individual experiments. Outlying datum points were handled by the Dixon criterion (33).

RESULTS

MICs and PAEs. MICs and the duration of the PAE are given in Table 1.

TABLE 1. MICs and duration of PAE for *S. aureus*, *P. aeruginosa*, and *E. coli*

Organism	Antibiotic	MIC (μg/ml)	Concn. ($n \times$ MIC)	PAE (h)[a]
S. aureus ATCC 25923	Dicloxacillin	0.25	2	1.5 ± 0.4
			4	2.3 ± 0.3
	Vancomycin	1	4	1.9 ± 0.4
	Rifampin	0.015	4	4.2 ± 0.6
	Ciprofloxacin	0.25	1	1.4 ± 0.7
			2	1.5 ± 0.1
			4	3.1 ± 0.9
E. coli ATCC 25922	Imipenem	0.125	1	−0.2 ± 0.1
			2	−0.2 ± 0.2
	Gentamicin	1	2	0.7 ± 0.5
			4	0.8 ± 0.1
	Tobramycin	2	1	0.7 ± 0.1
	Rifampin	16	4	6.9 ± 0.2
	Ciprofloxacin	0.015	1	1.2 ± 0.5
			2	1.6 ± 0.1
P. aeruginosa ATCC 27853	Imipenem	4	4	1.0 ± 0.2
			8	1.1 ± 0.4
	Tobramycin	0.5	2	1.4 ± 0.3
	Ciprofloxacin	0.25	1	1.0 ± 0.2
			2	3.3 ± 0.4
			4	3.4 ± 0.3

[a] Values are means ± SDs.

DNA synthesis. The growth curves and the [^{3}H]thymidine or [^{3}H]adenine incorporation curves for untreated control organisms during the logarithmic growth phase were linearly related ($r = 0.962$ for *E. coli* for the first 90 min of growth, $r = 0.940$ for *P. aeruginosa* for the first 270 min of growth, and $r = 0.999$ for *S. aureus* for the first 180 min of growth). After the untreated control organisms had been subjected to the drug removal procedure, the average rate of DNA synthesis per organism increased six- to sevenfold during logarithmic growth to a peak level at 90 min for the gram-negative bacilli and 180 min after the drug removal procedure for *S. aureus* (measured as counts per minute per CFU per minute [Fig. 1]). However, the amount incorporated differed depending on the organisms, being 10-fold higher in *S. aureus* than in *E. coli* and *P. aeruginosa*. For all three species the DNA synthesis activity declined again before the bacterial growth reached a plateau, beginning at ~5×10^7 CFU/ml.

Different DNA synthesis patterns were observed during the PAE phase, and these different patterns were dependent on both the organism and the drug. For *S. aureus* the cell wall-active agents dicloxacillin and vancomycin produced PAEs of 2 h (range, 1.8 to 2.5 h; Table 1). The patterns of DNA synthesis during the PAE were similar with these two antibiotics (Fig. 2). The synthetic activity was suppressed compared with that in the controls for the first 1.5 h, but prior to termination of the growth suppression a rapid synthesis of DNA was observed, culminating in a higher level than that in the untreated controls (Fig. 2). Similarly, after exposure to rifampin, inducing a PAE of ~4 h (Table 1), DNA synthesis remained consistently low during the PAE phase, but rapid DNA synthesis again commenced prior to regrowth. In general, the difference in the time to reach peak average synthesis per organism for exposed and control organisms reflected approximately the duration of the PAE.

In sharp contrast, a progressive dose-dependent increase in [^{3}H]thymidine incorporation was observed during PAEs induced by ciprofloxacin, commencing immediately after drug removal and reaching a peak at 90 to 180 min (Fig. 2).

The DNA synthesis of *E. coli* was minimally affected during the PAE phase after exposure to gentamicin and tobramycin but was markedly suppressed after exposure to rifampin (Fig. 3). As demonstrated for *S. aureus*, the PAE phase after rifampin exposure was characterized by an increase in DNA synthesis prior to and during regrowth.

On the other hand, imipenem induced a negative PAE of −0.2 h in *E. coli*, and in contrast to the suppressed DNA synthesis observed during the PAE phase induced by rifampin, after imipenem exposure DNA synthesis was actually enhanced in *E. coli* compared with that in control organisms.

Ciprofloxacin induced concentration-dependent PAEs of 1.2 to 1.6 h in *E. coli*. Similar to the observations made for *S. aureus*, a concentration-dependent increase in DNA synthesis during the PAE phase was observed compared with that in the controls (Fig. 3).

In contrast to *E. coli*, imipenem induced a significant PAE of

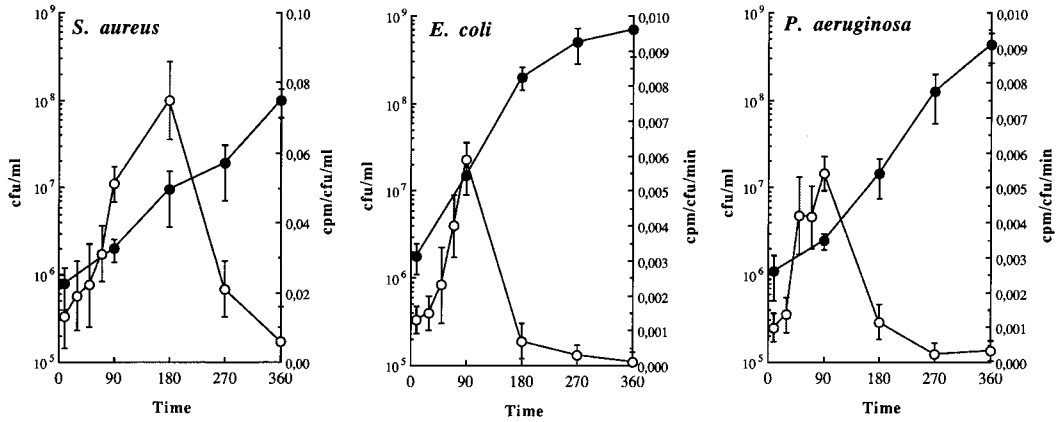

FIG. 1. Growth of unexposed *S. aureus* ATCC 25923, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 25783 measured by viability counting (closed symbols) and simultaneous rate of DNA synthesis per viable organism (open symbols). Points with bars represent means ± SDs.

1.0 to 1.2 h in *P. aeruginosa*. However, throughout the PAE phase the rate of DNA synthesis, as determined by [^{3}H]adenine incorporation, was not significantly different from that in controls after exposure of the organism to a concentration of 4× the MIC, and indeed, the rate was significantly higher than that in controls after exposure to a concentration of 8× the MIC (Fig. 4), akin to the observations made for *E. coli.*

During a PAE phase of 1.4 h (Table 1), tobramycin caused a marked suppression of [^{3}H]adenine incorporation in *P. aeruginosa* compared with that in unexposed control organisms (Fig. 4).

Ciprofloxacin induced a concentration-dependent PAE of 1.0 to 3.4 h in *P. aeruginosa* (Table 1). Similar to the observations made for *S. aureus* and *E. coli*, exposure to ciprofloxacin at a concentration of 4× the MIC was followed by a significant rise in [^{3}H]adenine incorporation. In spite of inducing PAEs, the lower concentrations of ciprofloxacin tested (1× and 2× the MIC) did not have a measurable impact on the [^{3}H]adenine incorporation compared with that in the controls (Fig. 4).

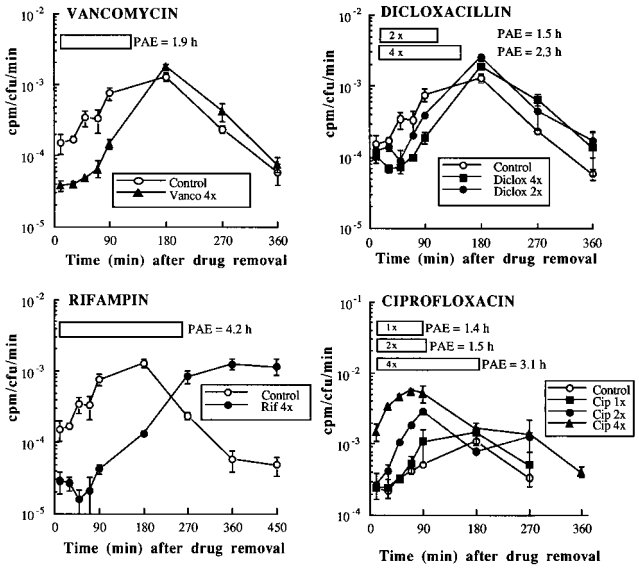

FIG. 2. DNA synthesis (*y* axis) during the PAE in *S. aureus* ATCC 25923 after exposure to dicloxacillin, vancomycin, ciprofloxacin, and rifampin compared with that in unexposed control organisms. Points with bars represent means ± SDs from two to four experiments, each of which was performed in quadruplicate. The horizontal bars at the top represent the duration of the PAE as determined by viable counts.

DISCUSSION

The PAEs induced in the present study were similar to the PAEs reported previously against the same bacterial species studied here (7). The data presented here indicate that DNA synthesis in bacteria, as determined by nucleotide precursor incorporation, is generally suppressed during the PAE compared with that in controls, with two important exceptions, however. Suppressed DNA synthesis was observed in *S. aureus* after exposure to dicloxacillin, vancomycin, and rifampin, in *E. coli* after exposure to rifampin, and in *P. aeruginosa* after exposure to an aminoglycoside. In contrast, DNA synthesis was relatively enhanced in the gram-negative bacilli after exposure to imipenem and in all three species after exposure to ciprofloxacin. However, the DNA synthesis in *E. coli* after exposure to tobramycin and gentamicin was only minimally affected, if at all.

DNA synthesis in unexposed *S. aureus* reached a peak ~90 min later than that in *E. coli*, reflecting the inherently slower growth rate. Furthermore, the incorporation of [^{3}H]thymidine (as counts per minute per CFU per minute) was 10 times higher for *S. aureus* than for *E. coli*, despite identical inocula and doses of radioactivity. The reasons for this difference are obscure. The weights of all three different bacterial organisms are similar. Lorian and colleagues (23) have demonstrated that gram-negative bacilli weigh 2×10^{-12} to 4×10^{-12} g and that a staphylococcus weighs 1.7×10^{-12} to 2×10^{-12} g. Accordingly, if the kinetics of nucleotide uptake in all three species is similar, the number of organisms per CFU may be 10-fold higher in staphylococci. Theoretically, the difference could represent a difference in the intracellular pool of endogenous thymidine. Alternatively, the kinetics of [^{3}H]thymidine uptake may depend on the bacterial species. This is supported by observations made by us and others (31) that *P. aeruginosa* fails to incorporate [^{3}H]thymidine but, instead, successfully incorporates [^{3}H]adenine.

Apart from these considerations, it is evident that changes in bacterial size, such as filamentation or the existence of multicellular organisms, can affect the results, since the increased biomass will not be reflected in the viability counting. We have previously studied ultrastructural alterations in *S. aureus* and *P.*

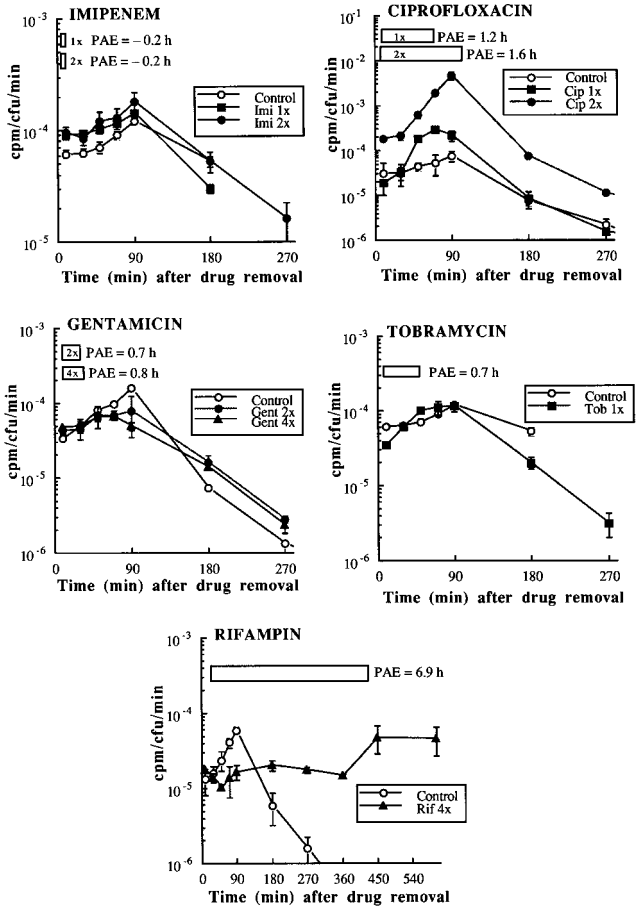

FIG. 3. DNA synthesis (*y* axis) during the PAE in *E. coli* ATCC 25922 after exposure to imipenem, gentamicin, tobramycin, rifampin, and ciprofloxacin compared with that in unexposed control organisms. Points with bars represent means ± SDs from two to three experiments, each of which was performed in quadruplicate. The horizontal bars at the top represent the duration of the PAE as determined by viable counts.

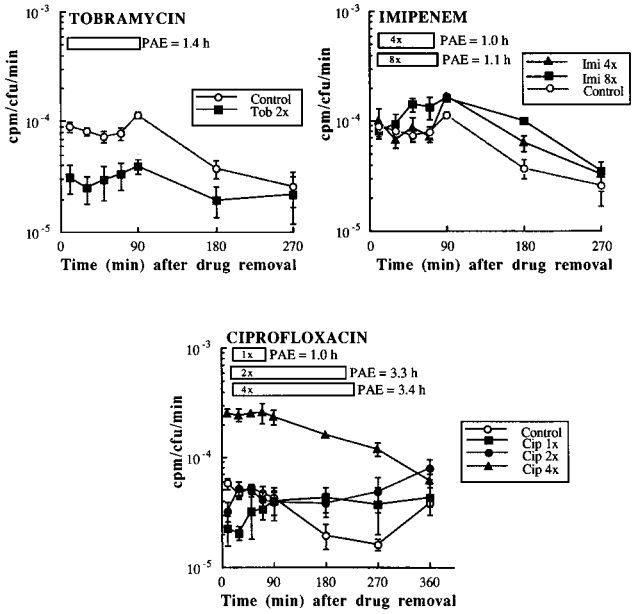

FIG. 4. DNA synthesis (*y* axis) during the PAE in *P. aeruginosa* ATCC 25783 after exposure to imipenem, tobramycin, and ciprofloxacin compared with that in unexposed control organisms. Points with bars represent means ± SDs from two to three experiments, each of which was performed in quadruplicate. The horizontal bars at the top represent the duration of the PAE as determined by viable counts.

aeruginosa during the PAE after exposure to several different antimicrobial agents (13). In that study, filamentation was not induced in *P. aeruginosa* after exposure to ciprofloxacin or imipenem. However, multicellular staphylococci were noted during the PAE after exposure to dicloxacillin, which could lead to underestimation of the level of suppression of DNA synthesis. As expected, we observed filamentation in *E. coli* during the PAE after exposure to ciprofloxacin. Importantly, however, the uncorrected counts per minute for *E. coli* (total counts per minute) was higher than that for controls, supporting a real increase in incorporation.

The mechanisms of the PAE are unknown, and few studies have focused on DNA synthesis in bacteria during this period of growth suppression (1, 14, 27). Investigations into the metabolic or intracellular events that occur during the PAE might provide an insight into this phenomenon and improve our understanding of the actions of antibiotics on bacteria. Odenholt and coworkers (27) demonstrated low metabolic activity in *Streptococcus pyogenes* during the PAE after exposure to penicillin by measuring [^{3}H]thymidine uptake during continuous precursor exposure. By using cumulative radiolabelled nucleoside precursor uptake, Barmada et al. (1) recently demonstrated that during tobramycin exposure, DNA, RNA, and protein syntheses in a clinical strain of *E. coli* were sequentially inhibited. However, following drug removal, both DNA and RNA syntheses resumed almost immediately, suggesting a possible dissociable binding of the drug to the nucleic acids, whereas protein synthesis did not resume until 4 h later. Likewise, following aminoglycoside exposure, we demonstrated only a minimal or no effect on DNA synthesis in *E. coli.*

In contrast, we demonstrated suppression of [^{3}H]adenine incorporation in *P. aeruginosa* after exposure to tobramycin, as opposed to the rather limited effect on *E. coli*. The reason for this difference is unclear, but it could pertain to a difference in the mechanism of action between species or the growth phases of the organisms. Theoretically, greater accumulation or entrapment of intracellular tobramycin in *P. aeruginosa* could account for the difference between the PAEs caused by the aminoglycoside in the two gram-negative bacilli (Table 1). Dubin and colleagues (9) have previously demonstrated that one of the late effects of streptomycin against *E. coli* is inhibition of DNA synthesis. Assuming a similar mechanism of action for different aminoglycosides, the suppression of DNA synthesis may occur only if a certain threshold of growth suppression (PAE) is reached, thus prolonging the time needed for the organisms to resume biosynthetic activity. Interestingly, the uptake of gentamicin during the PAE has been shown to be suppressed in *P. aeruginosa* (20).

A marked, dose-dependent enhancement in DNA synthesis was demonstrated in all three species tested during the PAE after exposure to ciprofloxacin. Other investigators have reported similar findings. Engle et al. (10) have shown that partial inhibition of DNA gyrase in *E. coli* is followed by a secondary increase in DNA synthesis. Similarly, Benbrook and Miller (2) demonstrated that an initial inhibition of DNA replication by norfloxacin (4× the MIC) in *P. aeruginosa* was followed by a secondary increase.

The observed enhancement in DNA synthesis after exposure to ciprofloxacin may represent induction of an SOS response

(36). The quinolones have been demonstrated to be potent inducers of the SOS response (29), which involves increased DNA repair activity. Quinolones bind to DNA gyrase and inhibit its supercoiling activity (11, 37). It has been postulated that complexes of gyrase and quinolones bind to DNA, resulting in inhibition of DNA replication and accumulation of single-stranded DNA, which is thought to stimulate increased repair activity (32). The fundamental components of the SOS response consist of a positive regulatory element (the *recA* gene) and a repressor element (the *lexA* gene) (36). Although the SOS response has been described in greatest detail in *E. coli* (36), genes with similar functions have been characterized in *P. aeruginosa* (21). In contrast, to our knowledge no studies have similarly focused on *S. aureus*, but evidence of the existence of RecA and LexA analog proteins in *Bacillus subtilis* has been provided, thereby demonstrating functional similarities between essential components of the DNA repair systems of widely divergent organisms (24). Theoretically, the increased level of DNA synthesis could therefore represent an increase in repair activity in all three bacterial species.

In contrast to our findings, Guan and colleagues (14) recently demonstrated suppression of DNA synthesis in *E. coli* during the PAE induced by sub-MICs of quinolones. This suppression continued for several hours after removal of the drugs and lasted longer than the PAE determined in the standard fashion. This discrepancy between different studies may be methodological, since we used concentrations of 1× to 4× the MIC, resulting in high levels of bactericidal activity, while Guan and associates (14) used sub-MICs to minimize bacterial death. While Guan et al. (14) used optical density to correct DNA for biomass, this approach was not feasible in our work because of the presence of high amounts of ghost cells and bacterial debris, a potential confounding factor. Apart from these considerations, the differences in the results might also be explained by distinct concentration-dependent morphological and biochemical changes as demonstrated by Diver and Wise (8) in *E. coli* after exposure to ciprofloxacin.

Imipenem is unique among the β-lactams because although it induces a PAE against *P. aeruginosa*, but it does so less readily than it does against other Gram-negative bacilli (6, 18, 28). The DNA synthesis curve over time after exposure of *P. aeruginosa* to imipenem (Fig. 4) was similar to that for control organisms, but the peak was higher than that for unexposed bacteria. Even though imipenem did not induce an actual PAE in *E. coli*, a similar pattern was observed, with the curves for control and unexposed organisms being similar. The relatively high rate of DNA synthesis observed after imipenem exposure in *E. coli* and *P. aeruginosa* is of interest and probably represents continued DNA replication and inhibition in bacterial septation and multiplication at the same time. Alternatively, it might represent a positive feedback on the DNA biosynthesis during interference with penicillin-binding proteins in the cell wall. Lorian and colleagues (22) have similarly shown that in *E. coli* the incorporation of thymidine is increased after 1 h of incubation in the presence of ampicillin. On the other hand, the DNA synthesis in *S. aureus* after dicloxacillin exposure was suppressed for a brief period corresponding to the initial PAE phase. The discrepancy might be explained by different β-lactam targets on different organisms that differ biochemically and functionally (3, 35).

In our experiments no predictable association with class of drug was found for the degree of suppression of DNA synthesis. The level of DNA synthesis per organism was generally resurrected shortly prior to the initiation of bacterial regrowth and started to decline at the time of logarithmic regrowth, i.e., prior to termination of the PAE phase. Thus, this most likely reflects resynthesis of DNA and initial cell division metabolism prior to cell separation, as measured by viable counts.

In the present study we have demonstrated a variable inhibition of DNA synthesis in bacteria during the PAE after exposure to several antibiotics, with the exception that synthetic activity after exposure to imipenem and ciprofloxacin was enhanced. Thus, in summary the DNA synthesis patterns differed depending on the organism-antibiotic combination, suggesting that different mechanisms underlie the PAE. Clearly, additional studies on bacterial metabolism during the PAE, including RNA, protein, and peptidoglycan syntheses, might also provide valuable information on the nature of this elusive microbiological phenomenon.

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