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Increased Production of Beta-Lactamase Under Anaerobic Conditions in Some Strains of *Escherichia coli*

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A simple biological assay to detect beta-lactamase activity exhibited by selected cultures of *Escherichia coli* was used to test enzyme production in cells incubated aerobically and anaerobically. Anaerobic incubation resulted in increased size of zones of drug inactivation by some beta-lactamase-producing strains. The beta-lactamase activity of cell lysates was determined iodometrically for aerobically and anaerobically grown cells. The specific beta-lactamase activity for anaerobically grown cells was three to five times greater than for aerobically grown cells. Beta-lactamase production was determined to be constitutive in all strains and to be plasmid mediated, as demonstrated by transfer to *E. coli* K-12 by conjugation.

Beta-lactamases (penicillinases) are enzymes that can open the beta-lactam ring of the penicillins and cephalosporins (12, 13, 15). Beta-lactamases play an important part in the resistance of many bacteria to beta-lactam antibiotics (15). The beta-lactamases produced by gram-positive bacteria are frequently extracellular and inducible and have a greater affinity for the penicillins than for the cephalosporins (1). The majority of beta-lactamases produced by gram-negative bacteria are cell bound, constitutive, and produced in much smaller quantities than those produced by gram-positive bacteria (15). Although beta-lactamase production is not the sole factor in resistance of gram-negative bacteria to beta-lactam antibiotics (13), there is a correlation between enzyme activity and level of resistance to various beta-lactam antibiotics (10, 14). There have been some reports of increased beta-lactamase production at different growth rates (2), at different growth temperatures (14), and in different growth phases (6). In this paper we present evidence for increased beta-lactamase activity under anaerobic conditions in some strains of *Escherichia coli*.

MATERIALS AND METHODS

Organisms. All *E. coli* strains, *Proteus morganii* 10950, *Pseudomonas aeruginosa* 10851, and *Staphylococcus aureus* 6538P were obtained from the stock culture collection at St. Francis Hospital, Wichita, Kans. *E. coli* K-12 C600-185NX (nalidixic acid resistant and plasmid free) was obtained from Stanley Falkow, University of Washington, Seattle.

Antibiotics. Penicillin G was provided by Eli Lilly and Co., Indianapolis, Ind., and ampicillin was provided by Bristol Laboratories, Syracuse, N.Y. Carbenicillin was purchased from Pfizer, New York, N.Y., and nalidixic acid came from Sterling-Winthrop Research Inst.

Detection of beta-lactamase activity. Beta-lactamase activity was detected biologically (18). *S. aureus* 6538P, susceptible to 0.03 μg of penicillin G per ml, was seeded in Mueller-Hinton agar (Difco) plates (final concentration of 10^7 colony-forming units per ml) containing 10 μg of penicillin G per ml. Plates were spot-inoculated with a 1/1,000 loop (10^6 colony-forming units) from an overnight culture of the organism being tested. After 24 h of incubation, halo growth of *S. aureus* around the colonies indicated beta-lactamase production. Control plates, not containing penicillin G, were used to examine inhibition of indicator *S. aureus* by the organism being tested.

Crude enzyme preparation. Cells were grown aerobically and anaerobically on plates of brain heart infusion agar (Difco). After 12 h of incubation, cells were washed off with potassium phosphate buffer (0.1 M, pH 5.9) and were harvested at 10,000 × *g* for 10 min at 4°C. Portions of cells (0.5 g) were weighed and suspended in 10 ml of 0.1 M potassium phosphate buffer (pH 5.9). Cells were broken by ultrasonic treatment for 2 min with a Bronwill Biosonik III ultrasonic disintegrator (Bronwill Scientific, Rochester, N.Y.), using ice-water cooling. Broken cells were centrifuged at 15,000 × *g* for 50 min at 2°C. Cell debris was discarded, and the supernatant was assayed for beta-lactamase activity.

Enzyme induction. Cells were grown overnight in 50 ml of Mueller-Hinton broth at 37°C. The culture was then diluted 1:10 in fresh Mueller-Hinton broth, and incubation was continued for 3 h. After this, it was divided into two parts. To one part, sufficient penicillin G was added to give a final concentration of 500 μg/ml (4, 5, 8); the other part served as a control. Incubation was then continued at 37°C with shaking, to allow induction to take place. The concentration of penicillin G used as inducer had no inhibitory effect on the growth of bacteria in liquid medium (minimal inhibitory concentrations >2,000 μg/ml). Cells were then

harvested for 10 min at 10,000 × *g* at 2°C and were resuspended in 1/20 their original volume of 0.1 M potassium phosphate buffer (pH 5.9).

Beta-lactamase assay. Beta-lactamase activity was determined by the microiodometric method of Sykes and Nordstrom (16), using 0.2 mM penicillin G as a substrate. All experiments were performed at room temperature.

Units of beta-lactamase. One unit of beta-lactamase is defined as the amount of enzyme that hydrolyzes benzylpenicillin at the rate of 1 μmol/min at room temperature at pH 5.9. Specific beta-lactamase activity was defined as the units of beta-lactamase per milligram of protein in the enzyme solution.

Conjugation experiments. Cultures of donor and recipient strains were grown overnight in brain heart infusion broth. One milliliter of overnight culture was added to 100 ml of fresh brain heart infusion broth and incubated at 35°C with shaking until the absorbance was 0.52 at a wavelength of 550 nm for the donor strain (approximately 10^8 colony-forming units per ml) and 1.5 for the recipient strain (approximately 5×10^8 colony-forming units per ml). One-milliliter volumes of donor and recipient strains were mixed and incubated without agitation for 18 h at 35°C in a 25-ml Erlenmeyer flask as a mating mixture. Samples (0.1 ml) of mating mixture were plated on Mueller-Hinton agar plates containing (i) nalidixic acid (20 μg/ml) plus ampicillin (50 μg/ml) or (ii) nalidixic acid (20 μg/ml) plus carbenicillin (50 μg/ml). Spontaneous mutants in parental strains were estimated by plating donor and recipient strains on each of the above-mentioned plates.

Protein determination. Protein determination of cell lysates was routinely carried out by the method of Lowry et al. (7) with bovine serum albumin (fraction V) as the standard. All spectrophotometric measurements were made with a Beckman ACTA CIII spectrophotometer.

RESULTS

Preliminary screening for beta-lactamase activity. *S. aureus* 6538P was seeded in Mueller-Hinton agar plates containing 10 μg of penicillin G per ml. Duplicate plates were spot inoculated with a 1/1,000 loop from an overnight culture of the strain to be tested. One of the plates was incubated aerobically, and the other was incubated anaerobically at 37°C. After 24 h plates were examined for halo growth of *S. aureus* around the inoculum. Some strains were found to produce a larger zone of halo growth under anaerobic conditions than under aerobic conditions (Fig. 1). The relative diffusion of a fixed amount of beta-lactamase under aerobic and anaerobic conditions was compared by dispensing 50 μl of an enzyme preparation, with activity of 2 U/ml, into wells made in agar plates containing penicillin G and *S. aureus* 6538P (as described above). The plates were examined for halo growth of indicator *S. aureus* after 24 h of aerobic and anaerobic incubation; there was no difference in the size of zones of halo growth of *S. aureus* 6538P around the wells under aerobic and anaerobic conditions.

Beta-lactamase formation at different phases of growth. Cells were grown in brain heart infusion broth at 37°C with shaking. Samples (10 ml) were taken every 2 h and beta-lactamase activity was determined after ultrasonic disruption. Maximum enzyme activity occurred about 12 h after initiation of growth (Fig. 2). After 24 h there was a decrease in beta-lactamase activity. In all strains beta-lactamase was produced constitutively, and attempts to increase the enzyme activity by induction with penicillin G were fruitless (Table 1). *Proteus morganii* 10950 and *Pseudomonas aeruginosa* 10851 were used as positive controls in induction experiments and showed a 25- and 14-fold increase in beta-lactamase activity, respectively, in the presence of 500 μg of penicillin G per ml (Table 1).

Effect of anaerobic conditions on beta-lactamase activity. Strains which produced an increased zone of halo growth under anaerobic conditions in the beta-lactamase screening test were grown on brain heart infusion agar plates aerobically and anaerobically. Since cells grew more slowly under anaerobic conditions, more

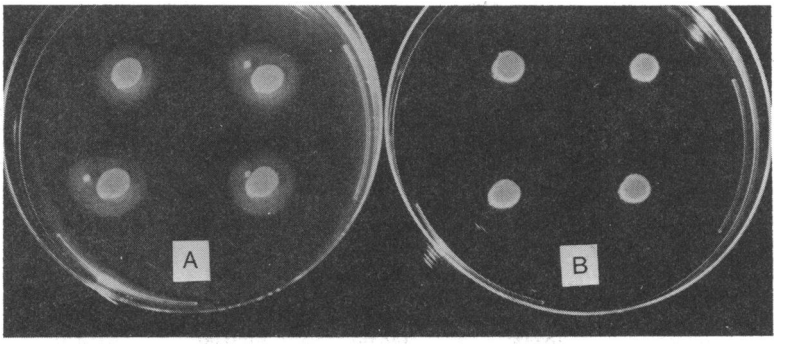

FIG. 1. *Screening of beta-lactamase activity of E. coli 10939 under (B) aerobic and (A) anaerobic conditions.*

plates were required to yield the same cell harvest under anaerobic conditions. After 12 h of incubation, cells were washed off and disrupted, and the beta-lactamase activity of cell-free supernatants was determined. Specific beta-lactamase activity of anaerobically grown cells was three to five times more than that of aerobically grown cells (Table 2).

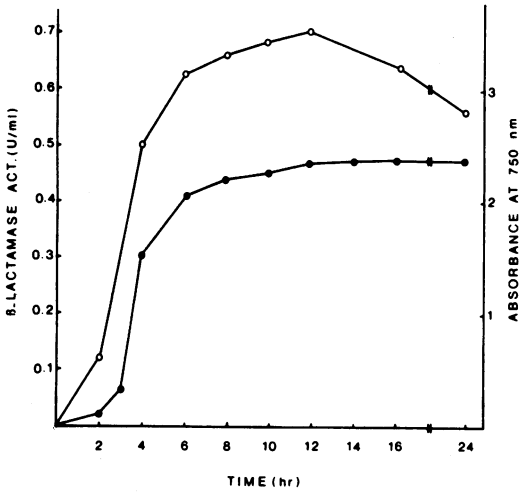

FIG. 2. *Beta-lactamase formation at different phases of growth in E. coli F-82. Cells were grown at 37°C with shaking. Samples (10 ml) were taken every 2 h and absorbance at 750 nm (●) and beta-lactamase activity of broken cells (○) were determined.*

TABLE 1. *Beta-lactamase activity of E. coli strains in the presence and absence of inducer*

Strain	Beta-lactamase activity (U)	
	Induced	Not induced
E. coli G-95	1.38	1.39
E. coli G-105	1.12	1.10
E. coli F-54	2.04	2.03
E. coli F-82	1.45	1.45
E. coli 26105	1.98	1.95
E. coli 10939	3.23	3.25
P. morganii 10950	4.80	0.19
P. aeruginosa 10851	2.10	0.15

Transferability of beta-lactamase genes. All strains that were determined to have increased beta-lactamase activity under anaerobic conditions were examined for transferability of beta-lactamase genes. All strains were capable of transferring their beta-lactamase genes to the recipient *E. coli* K-12 C600-185NX by conjugation. *E. coli* strains 10897, 10854, and 26457 were not capable of transferring their beta-lactamase genes to *E. coli* K-12 C600-185NX. These strains did not show any increase in the sizes of zones of halo growth of *S. aureus* in the beta-lactamase screening test when incubated under anaerobic conditions. The transferability of beta-lactamase production to *E. coli* K-12 by conjugation suggests that the beta-lactamase genes are carried on plasmids. Frequencies of transfer of ampicillin and carbenicillin resistance are shown in Table 3. Beta-lactamase production by *E. coli* K-12 transconjugants was confirmed qualitatively with the microiodometric technique. Transduction of beta-lactamase genes was ruled out by demonstrating that no resistant colonies could be isolated when bacteria-free filtrates of exponential-phase cultures of the donors were incubated with nalidixic acid-resistant *E. coli* K-12 in the same manner as described for conjugation (9). Examination of control plates established that no spontaneous mutants of parental strains were obtained in the inoculum used (0.1 ml from 10^{-1} dilution).

DISCUSSION

The increased sizes of zones of halo growth of the indicator organism under anaerobic condi-

TABLE 3. *Frequencies of transfer of ampicillin and carbenicillin resistance to E. coli K-12*[a]

Donor strain	Ampicillin	Carbenicillin
G-95	8.5×10^{-1}	10.2×10^{-1}
G-105	4.0×10^{-2}	7.0×10^{-1}
F-82	2.5×10^{-5}	3.5×10^{-5}
F-54	9.5×10^{-4}	9.7×10^{-3}
26105	1.8×10^{-3}	2.3×10^{-2}
10939	1.4×10^{-3}	4.3×10^{-4}

[a] Calculated as number of recombinants per recipient.

TABLE 2. *Beta-lactamase activity of E. coli strains grown under aerobic and anaerobic conditions*

E. coli strain	Beta-lactamase activity[a]		Protein concn[b]		Enzyme sp act	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
G-95	7.35	27.30	131.10	140.20	0.056	0.194
G-105	4.64	23.08	97.60	104.00	0.047	0.221
F-82	6.90	40.40	119.00	135.00	0.058	0.299
F-54	12.40	42.28	127.60	140.20	0.097	0.301
26105	7.66	46.20	107.00	129.00	0.071	0.358
10939	32.60	182.00	148.60	160.00	0.220	1.137

[a] Enzyme units per gram (wet weight) of cells.
[b] Milligrams of protein per gram (wet weight) of cells.

tions (Fig. 1) suggest that either the liberation of beta-lactamase into surrounding medium was increased, or the synthesis of the enzyme was increased. The increased specific beta-lactamase activity in crude lysates from anaerobically grown, washed cells suggests the second possibility. Having determined that more beta-lactamase is produced after 12 h of incubation under anaerobic conditions than under aerobic conditions, we examined the rate of beta-lactamase production under aerobic conditions to determine whether it decreased during the first 12 h of incubation (Fig. 2). It was found that the total amount of beta-lactamase per culture increased steadily during the first 12 h of aerobic growth; therefore the difference detected between aerobically and anaerobically grown cells could not be due to reduced production of beta-lactamase under aerobic incubation conditions.

Beta-lactamase production was determined to be constitutive and transmissible in all six strains that showed the anaerobic incubation effect. Studies are presently under way in this laboratory to isolate and further characterize these plasmids.

It has been shown that there is a linear correlation between beta-lactamase activity and the level of resistance of bacteria to penicillins (10, 13, 15). Since conditions in the mammalian gastrointestinal tract are mainly anaerobic (3), and the effect of beta-lactamases on penicillins under anaerobic conditions (in vivo) is known (11), it may be that increased beta-lactamase production under anaerobic conditions could provide an extra margin of resistance to the organisms against beta-lactam antibiotics. This might explain the dichotomy often observed between the in vitro and in vivo activity of beta-lactam antibiotics (17).

Engberg and Nordstrom (2) have shown that the initiation mass of plasmid replication is proportional to the growth rate of the host cell; with slow growth rates the number of plasmid copies per cell was increased, and there was a correlation between gene dosage and beta-lactamase biosynthesis. We found that under anaerobic conditions cells grew more slowly than under aerobic conditions; we postulate that there may be an increase in the number of plasmid copies per cell under anaerobic conditions, and that this increase may account for the observed increase in beta-lactamase activity.

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