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D-Lactic Acid Production as a Monitor of the Effectiveness of Antimicrobial Agents

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Most bacteria at an infection site obtain energy by the breakdown of glucose via microaerophilic or anaerobic pathways and in the process yield various end products. In this study, production of D-lactic acid by *Staphylococcus aureus* and *Escherichia coli* was correlated with glucose utilization by bacteria during exposure to antibiotics at subinhibitory, inhibitory, and suprainhibitory concentrations. D-Lactic acid production was further correlated with production of a tissue-destroying enzyme, hyaluronidase, by *S. aureus*. For *E. coli*, all agents tested showed dose-related bacterial killing, with the most noticeable being with ampicillin, piperacillin, and ciprofloxacin. Imipenem, ciprofloxacin, and chloramphenicol had the most dose-related effects on D-lactic acid production. With few exceptions, hyaluronidase production correlated well with D-lactic acid production in *S. aureus*. Subinhibitory concentrations of erythromycin and clindamycin effectively decreased accumulation of D-lactic acid and hyaluronidase. Determination of D-lactic acid production may perhaps serve as a means of independently monitoring the effects of antimicrobial agents on bacterial metabolic activity, which is an important aspect of antimicrobial action that remains relatively unexplored.

The interactions of antibiotics and bacteria have been studied extensively (4, 8, 14, 21). However, the ability of an antimicrobial agent to halt the metabolic activity of an organism and, hence, to arrest the production and excretion of tissue-damaging bacterial products has been studied less extensively (16, 19, 22).

Knowledge about alterations in metabolic activity during antibiotic exposure can be potentially useful. While use of bacteriostatic antibiotics such as chloramphenicol, clindamycin, or lincomycin has resulted in an unusual degree of success in treating a wide variety of human infections (10, 17), chloramphenicol is known to be unsuccessful against *Staphylococcus aureus* endocarditis (9, 13), whereas clindamycin has been shown to produce a good success rate (17). Therefore, not all bacteriostatic antibiotics produce the same antimicrobial effect.

In most areas of the body in which bacteria are found to be invasive, a microaerophilic or a near-anaerobic environment is present or is established soon after the infection ensues (12). In this anaerobic environment, almost all the pathogenic bacteria generate energy for survival and proliferation while utilizing in part the available carbohydrates to yield D-lactic acid as an end product (11).

The elevation of L-lactate in body fluid during bacterial infections (mostly endogenous or of host origin) has already been established (2). Elevation of D-lactic acid (an exclusively bacterial isomer) in blood of animals with experimental infections has been reported previously (18). Another important end product is the extracellular enzyme hyaluronidase which is produced by *S. aureus* as well as *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Clostridium perfringens*. This enzyme was originally called "spreading factor" or "mucinase" and was thought to be important during the early stages of infection (1).

This in vitro study was designed to detect the effect of exposure to subinhibitory, inhibitory, and suprainhibitory concentrations of commonly used antimicrobial agents on the production of D-lactic acid by *Escherichia coli* and *S. aureus* and hyaluronidase by *S. aureus*. Antibiotics with actions at a variety of sites and with a variety of mechanisms of action were chosen for these experiments.

MATERIALS AND METHODS

Antibiotics. Antibiotic laboratory test powders were obtained from the manufacturers as follows: ampicillin, Bristol Laboratories, Syracuse, N.Y.; cefotaxime, Hoechst-Roussel Pharmaceuticals, Inc., Sommerville, N.J.; chloramphenicol, Parke-Davis, Morris Plains, N.J.; clindamycin, Upjohn Co., Kalamazoo, Mich.; ciprofloxacin, Miles Laboratories, West Haven, Conn.; erythromycin, Abbott Laboratories, Abbott Park, Ill.; gentamicin, Schering Corp., Kenilworth, N.J.; imipenem, Merck Sharp & Dohme, Rahway, N.J.; oxacillin, Bristol Laboratories; penicillin, Wyeth Laboratories, Philadelphia, Pa.; piperacillin, Lederle Laboratories, Pearl River, N.Y.; rifampin, CIBA-GEIGY Corp., Summit, N.J.; tetracycline, Pfizer Laboratories, New York, N.Y.; and vancomycin, Eli Lilly & Co., Indianapolis, Ind. All antibiotics except ciprofloxacin and rifampin were dissolved in 0.1 M phosphate buffer (pH 7.0) at a concentration of 1 mg/ml as stock solution; ciprofloxacin and rifampin were dissolved in dimethyl sulfoxide. All stock solutions were prepared and used the same day by further diluting them to the desired concentrations in prereduced chopped meat glucose broth (Scott Laboratories, Inc., Fiskeville, R.I.).

Bacteria. The bacterial strains studied included *E. coli* ATCC 25922, *S. aureus* ATCC 29213, and a clinical isolate of *S. aureus*. The strains from the American Type Culture Collection (ATCC; Rockville, Md.) were chosen for their established responses to a variety of antimicrobial agents. The clinical isolate was chosen for its susceptibility to ampicillin and penicillin and for its ability to produce high levels of hyaluronidase.

MIC determinations. MICs were determined in chopped

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meat glucose broth under anaerobic conditions. Antibiotics were serially diluted twofold in a volume of 1 ml in prereduced clear chopped meat glucose broth. An inoculum of 10^7 CFU of the test organism per ml was added, and the tubes were incubated anaerobically for 18 to 24 h. Standard MICs were produced by using Mueller-Hinton broth at an inoculum size of 5×10^5 CFU/ml under aerobic incubation conditions. With gentamicin, the Mueller-Hinton broth was supplemented with divalent calcium and magnesium cations.

Test conditions for D-lactic acid and hyaluronidase production and glucose utilization. Prereduced chopped meat glucose broth with and without antibiotics was used. Antibiotic concentrations used were subinhibitory (1/4× the MIC), inhibitory (4× the MIC), and suprainhibitory (16× the MIC). Organisms were added to each tube to give a final turbidity equal to a no. 0.5 McFarland turbidity standard. The tubes were individually reduced and placed in an anaerobic environment. Sampling times included 0, 3, 6, 12, and 24 h. At each time point a portion was removed for quantitative bacterial count and D-lactic acid and glucose determinations. In experiments involving *S. aureus*, hyaluronidase activity was also assayed. Processing and preservation of samples in the individual assays are described below. A growth control without antibiotic was included for each organism. Quantitative counts were determined by serial dilution and were subcultured onto blood agar plates.

D-Lactic acid assay. To each sample was added two volumes of chilled 7% perchloric acid. The mixture was centrifuged at 1,500 × *g* for 20 min. The supernatants were assayed as described by Smith et al. (18). D-Lactate dehydrogenase, assay buffer (pH 9.2), and NAD (Sigma Chemical Co., St. Louis, Mo.) were added to all test samples. Individual sample blanks containing only the sample and water were used. Simultaneous samples with known amounts of D-lactic acid (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) were also tested to generate a standard curve. All tubes were incubated in a water bath at 35°C for 1 h, and the A_{340} was determined with a UV-Vis model 300N spectrophotometer (Gilford Systems, Oberlin, Ohio). The sample D-lactic acid concentrations were determined by interpolation of points generated from the standard curve.

Glucose utilization assay. Glucose concentrations were determined by using the method of *o*-toluidine for hexoses (3). Samples used for determination of D-lactic acid were also assayed for glucose concentrations. The *o*-toluidine reagent was obtained from Sigma Chemical Co. The sample hexose concentrations (only glucose was initially present in the medium) were determined by interpolation of the curve generated from known standard concentrations of glucose.

Hyaluronidase determinations. Samples for hyaluronidase testing were frozen at −70°C prior to testing. Hyaluronidase production was determined by the procedure of Dorfman (5). In this assay, hyaluronic acid (human umbilical cord; Sigma Chemical Co.) was dissolved in reagent A (0.3 M sodium phosphate [pH 5.35] at 37°C), which was prewarmed to 37°C prior to use. Unknown samples diluted in reagent B (0.02 M sodium phosphate containing 0.45% sodium chloride and 0.01% bovine serum albumin [pH 7.0] at 37°C) were added to the hyaluronic acid substrate and incubated for 45 min in a water bath at 37°C. The reagent blank consisted of reagents A and B. After incubation, 0.1% bovine serum albumin in 0.1 M sodium acetate (pH 3.75 at 25°C) (reagent C) was added to all tubes. Tubes were inverted twice and allowed to stand at room temperature for an additional 10 min. The percent transmission at 660 nm was determined against that for water. The change in percent transmission was determined for samples and standards by subtracting the percent transmission of the reagent blank from the percent transmission of the sample. The final concentration of hyaluronidase in unknown samples was determined from interpolation of a standard curve generated by using known units of hyaluronidase activity (sheep testes; Sigma Chemical Co.).

TABLE 1. MICs determined by using an inoculum of 10^7 CFU/ml in chopped meat glucose broth

Antibiotic	MIC (μg/ml) for:		
	E. coli ATCC 25922	*S. aureus* ATCC 29213	*S. aureus* (clinical isolate)
Ampicillin	8.0 (4.0)[a]	ND[b]	0.25 (0.06)
Penicillin	ND	ND	0.06 (0.06)
Cefotaxime	0.5 (0.12)	0.5 (1.0)	ND
Piperacillin	8.0 (4.0)	ND	ND
Oxacillin	ND	0.25 (0.25)	ND
Imipenem	2.0 (0.12)	0.06 (0.03)	ND
Ciprofloxacin	0.125 (0.01)	1.0 (0.25)	ND
Gentamicin	>16 (1.0)	>16 (1.0)	ND
Chloramphenicol	8.0 (8.0)	16.0 (8.0)	ND
Clindamycin	ND	0.5 (0.12)	ND
Erythromycin	ND	2.0 (0.25)	ND
Vancomycin	ND	4.0 (1.0)	ND
Tetracycline	4.0 (2.0)	0.5 (1.0)	ND
Rifampin	ND	0.008 (0.008)	ND

[a] Results in parentheses were determined by using a standard inoculum size and Mueller-Hinton broth.
[b] ND, Not done.

Antibiotic assay. Antibiotic levels in the incubation broth were assayed at 24 h of incubation. Bioassays were performed with *Bacillus subtilis* ATCC 6633, *S. aureus* ATCC 29213, or *E. coli* ATCC 25922 as the indicator organism, according to the antibiotic used (see Table 4).

RESULTS

MIC determinations. Since all assays and incubations of organisms were performed anaerobically in chopped meat glucose broth, the MICs were determined under similar conditions and with a comparable inoculum size. A summary of the results is shown in Table 1. The MIC results by use of anaerobic incubation were within 2 dilutions of the results obtained by use of standard procedures, with the following exceptions. Antibiotics with MICs which were greater than 2 dilutions higher included gentamicin for *S. aureus* and *E. coli*, imipenem and ciprofloxacin for *E. coli*, and erythromycin for *S. aureus*. Imipenem has the potential to be destroyed by reducing agents in the medium, but from the observed MICs under aerobic and anerobic conditions, significant loss of activity did not occur with staphylococci. As expected, gentamicin had poor antibacterial activity under these conditions and was purposely included as a control for the experiments performed under anaerobic conditions.

Bacterial viability. Uninoculated chopped meat glucose broth had no detectable D-lactic acid. The initial bacterial density of both organisms was approximately 10^7 CFU/ml. For *E. coli* exposed to a subinhibitory antibiotic concentration (1/4× the MIC), bacterial viability was unaffected. At near inhibitory (4× the MIC) and suprainhibitory (16× the MIC) concentrations, ampicillin, piperacillin, and ciprofloxacin produced significantly reduced bacterial viability (Table 2). For *S. aureus*, no reduction in CFU was seen at subinhibitory levels; however, at near inhibitory concentrations (4× the MIC), reductions were seen with oxacillin and

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TABLE 2. Inhibition of D-lactic acid accumulation in *E. coli*

Antibiotic	% of control at the following multiple of the MIC and the indicated time:							
	0×		1/4×		4×		16×	
	12 h	24 h	12 h	24 h	12 h	24 h	12 h	24 h
None (control)	42.0[a]	45.2[a] (9.2)[b]						
Ampicillin			39.7	101 (7.2)	0.8	18.6 (5.1)	1.3	0.9 (3.1)
Cefotaxime			49.6	109 (6.0)	2.9	5.4 (6.4)	2.5	5.5 (6.2)
Piperacillin			42.5	101 (6.6)	4.4	5.0 (4.1)	1.1	1.4 (4.1)
Imipenem			9.9	7.6 (8.3)	0.7	1.1 (7.5)	0.3	1.0 (6.2)
Ciprofloxacin			4.9	46.7 (7.5)	1.5	2.7 (2.0)	1.8	2.8 (2.8)
Tetracycline			10.9	115 (8.8)	1.2	3.6 (7.1)	0.7	1.8 (6.8)
Chloramphenicol			0.9	50 (8.5)	0.9	1.1 (7.2)	0.8	1.3 (6.1)
Gentamicin			94.2	97.6 (9.1)	19.2	110 (8.5)	1.4	3.7 (3.3)

[a] Control data are expressed as millimolar.
[b] Values in parentheses are $\log_{10}$ CFU per milliliter.

cefotaxime, with greater than a 2 $\log_{10}$ decrease (Table 3). At a suprainhibitory concentration (16× the MIC), more dramatic reductions were seen. The 24-h CFU counts were 5.4 for oxacillin, 4.1 for cefotaxime, 5.3 for imipenem, 5.4 for vancomycin, and 4.1 for ciprofloxacin. Gentamicin failed to inhibit the growth of either organism tested at subinhibitory and inhibitory concentrations and was included as a control since its effectiveness is known to be reduced in an anaerobic environment (15). Antibiotic destruction during the incubation period was assessed (Table 4). All antibiotics except imipenem and rifampin had greater than 50% activity at the end of a 24-h incubation; imipenem is known to be physically unstable and rifampin had only 29.3% activity remaining.

***E. coli* D-lactic acid production.** The rates of D-lactic acid accumulation and glucose utilization for a control culture containing no antibiotics are shown in Fig. 1. D-Lactic acid production followed glucose utilization during the logarithmic phase of growth at 3 to 12 h before reaching a plateau after 12 h. When organisms were exposed to antibiotics at subinhibitory concentrations, there was an initial inhibition of D-lactic acid accumulation at 12 h for all agents except gentamicin; however, at 24 h the amounts of D-lactic acid produced closely approximated that of the control except for imipenem, ciprofloxacin, and chloramphenicol, which showed decreased production (Table 2). At suprainhibitory concentrations, all agents inhibited D-lactic acid accumulation. Despite its bactericidal activity, ampicillin was not as effective as the other cell wall-active antibiotics in inhibiting D-lactic acid accumulation. Gentamicin was totally ineffective in inhibiting D-lactic acid production at subinhibitory and near inhibitory concentrations, as was expected because of its lack of activity under anaerobic conditions (15).

***S. aureus* D-lactic acid production.** The rates of D-lactic acid and hyaluronidase accumulation and glucose utilization by *S. aureus* ATCC 29213 during normal growth are shown in Fig. 2. Accumulation of D-lactic acid followed utilization of glucose. Hyaluronidase accumulation reached a plateau after 12 h. With increasing antibiotic concentrations, less D-lactic acid was produced (Table 3). The initial inhibition of lactic acid accumulation at 12 h was more obvious at 24 h. At 12 h, cefotaxime, erythromycin, clindamycin, and chloramphenicol were very effective in reducing D-lactic acid pro-

TABLE 3. Inhibition of D-lactic acid and hyaluronidase accumulation in *S. aureus*

Organism and antibiotic	% of control at the following multiple of the MIC and the indicated time:									
	1/4× the MIC					4× the MIC				
	D-Lactic acid		Hyaluronidase		$\log_{10}$ CFU/ml (24 h)	D-Lactic acid		Hyaluronidase		$\log_{10}$ CFU/ml (24 h)
	12 h	24 h	12 h	24 h		12 h	24 h	12 h	24 h	
S. aureus ATCC 29213										
Oxacillin	76.0	95.8	63.1	97.0	8.2	1.4	3.3	0	0	5.5
Cefotaxime	42.4	56.2	14.4	63.5	8.2	1.9	3.0	0	0	5.3
Imipenem	117	110	92.0	92.5	8.5	4.5	3.4	0	0	6.2
Vancomycin	72.1	95.9	58.3	99.4	8.5	1.0	2.2	0	41.1	6.3
Erythromycin	12.8	39.6	0	0	8.1	1.5	1.8	0	0	7.4
Clindamycin	14.0	11.3	0	0	8.1	6.8	6.3	0	0	7.5
Tetracycline	61.3	70.0	104	102	8.6	1.6	2.8	0	0	7.5
Gentamicin	75.0	84.5	63.8	91.8	8.5	3.4	22.8	0	100	8.3
Chloramphenicol	4.7	12.6	0	103	8.3	2.1	2.1	0	0	7.2
Ciprofloxacin	108	90.9	101	110	8.6	3.4	3.1	0	11.9	7.2
Rifampin	83.6	120	25.5	66.4	8.5	0.4	3.5	0	0	6.1
Staphylococcus aureus clinical isolate										
Ampicillin	61.8	75.0	29.0	44.8	6.4	5.0	8.8	0	0	6.3
Penicillin	79.0	72.9	78.2	89.0	7.4	6.6	9.2	0	0	6.3

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TABLE 4. Antibiotic activity remaining in chopped meat glucose broth after 24 h

Antibiotic	Indicator organism	Concn used (μg/ml)	% Remaining at 24 h
Ampicillin	*B. subtilis* ATCC 6633	20	50.6
Penicillin	*B. subtilis* ATCC 6633	20	76.4
Cefotaxime	*E. coli* ATCC 25922	50	77.7
Piperacillin	*B. subtilis* ATCC 6633	75	77.9
Oxacillin	*B. subtilis* ATCC 6633	50	69.1
Clindamycin	*S. aureus* ATCC 29213	15	84.2
Erythromycin	*S. aureus* ATCC 29213	20	77.2
Tetracycline	*S. aureus* ATCC 29213	10	89.6
Vancomycin	*B. subtilis* ATCC 6633	25	65.8
Gentamicin	*B. subtilis* ATCC 6633	5	55.3
Imipenem	*B. subtilis* ATCC 6633	25	<1.0
Chloramphenicol	*B. subtilis* ATCC 6633	20	54.1
Ciprofloxacin	*B. subtilis* ATCC 6633	5	53.0
Rifampin	*S. aureus* ATCC 29213	2	29.3

duction, even at only 1/4× the MIC, with D-lactic acid concentrations being 42.4, 12.8, 14.0, and 4.7% of control values, respectively. Data for 16× the MIC are not shown and were comparable to the 4× MIC data. As with *E. coli*, gentamicin failed to have any antimicrobial effect on organism viability, although a reduction in D-lactic acid production was noted.

Glucose utilization. Glucose utilization preceded D-lactic acid production, as shown in Fig. 1 and 2. This probably indicates a tendency of the organisms to shift to a higher level of D-lactic acid production as the culture grows older. There was a good correlation of the amount of D-lactic acid accumulated and the amount of glucose utilized. For *E. coli*, the most dramatic reductions were seen with imipenem (51.4% of the available glucose was used when tested at 1/4× the MIC) and ciprofloxacin (47.8% of the glucose was utilized). Gentamicin failed to inhibit glucose utilization. For *S. aureus*, the most dramatic reductions in glucose utilization were seen with erythromycin and clindamycin, with 38.3 and 24.8% of glucose utilized, respectively, when it was exposed for 24 h to subinhibitory antibiotic concentrations (data not shown).

***S. aureus* hyaluronidase production.** Hyaluronidase production was halted by erythromycin and clindamycin even at

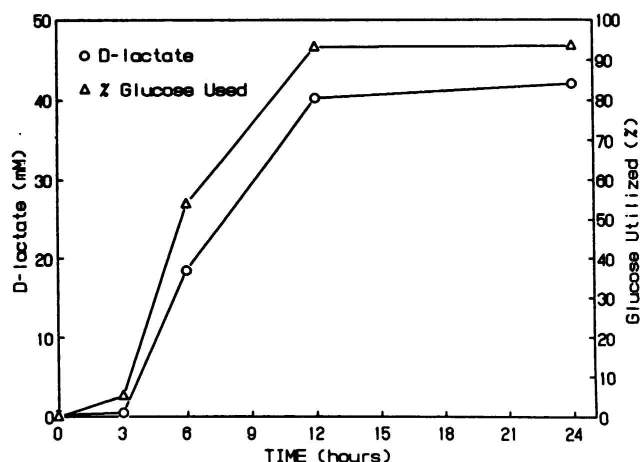

FIG. 1. D-Lactic acid accumulation and glucose utilization by *E. coli* ATCC 25922 during normal bacterial growth.

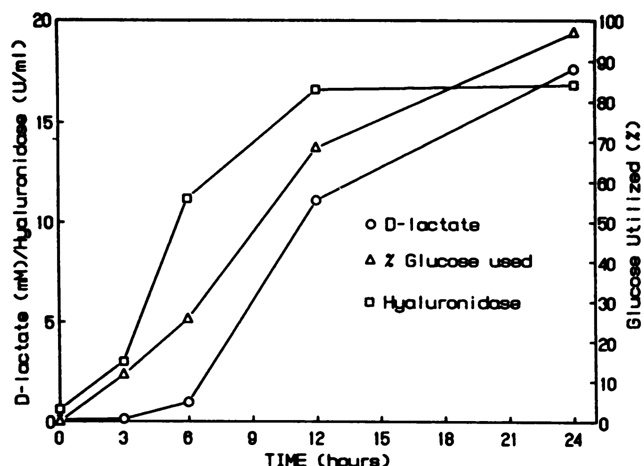

FIG. 2. D-Lactic acid accumulation, hyaluronidase accumulation, and glucose utilization by *S. aureus* ATCC 29213 during normal bacterial growth.

subinhibitory concentrations (Table 3). At near inhibitory concentrations (4× the MIC), only vancomycin (41.1% of the control value) and gentamicin (100% of the control value) failed to markedly inhibit hyaluronidase production. With the exceptions indicated above, D-lactic acid production correlated well with hyaluronidase production.

DISCUSSION

The major mechanism of energy metabolism for facultative bacteria under anaerobic conditions is fermentation (11). Glucose and other carbohydrates are broken down to pyruvate, and at that juncture, several enzymatic pathways can be used to convert pyruvate to various end products to generate additional energy for the microorganism. For most organisms that can cause human infections, the pathways primarily include lactic acid, mixed acid, butanediol, and butyric acid as end products (20). Measurement of the rates of production of these end products would more accurately define the metabolic integrity of the microorganisms.

In order to cause disease or tissue injury, microorganisms must have the ability to acquire energy to synthesize mediators of invasion, such as hydrolytic enzymes or toxins directed against specific tissues. Such comprehensive measurements are impractical for monitoring the effects of an antimicrobial agent on any particular microorganisms. Measurements of L-lactic acid or ethanol may produce some insight into the in vitro effects of antimicrobial agents on the bacterium, but they cannot be extended to in vivo studies because these two metabolites are easily converted to other compounds by host enzymes. Although one can directly measure the glucose utilization rate in vitro after exposure to an antibiotic, there are other hexoses that are utilized, and this can be measured only with difficulty during infection. For each antibiotic and microorganism, the amount of D-lactic acid produced in relation to the amount of glucose utilized can be determined in vitro. As can be seen in Fig. 1 and 2, a good correlation exists between the amount of D-lactic acid produced and the amount of glucose utilized in a time course study. Hence, if a metabolite were to be chosen to monitor antimicrobial action on bacterial metabolism in vitro and in vivo, it should be D-lactic acid because it is only produced by lower organisms and because it is easy to measure.

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The effects of various antimicrobial agents on the metabolic integrity of *E. coli* and *S. aureus* were examined by monitoring D-lactic acid production. In *E. coli*, imipenem, ciprofloxacin, and chloramphenicol were effective in inhibiting D-lactic acid production even at subinhibitory concentrations, whereas in *S. aureus*, erythromycin and clindamycin were effective at subinhibitory concentrations. A concentration-dependent inhibition of D-lactic acid production by the several antibiotics studied was observed, and this agrees with earlier observations that certain antibiotics can become more effective when the concentrations are raised above the MIC (6, 7).

The data reported here indicate that antibiotics have additional effects on microorganisms which have not yet been explored. This new area of research includes the effects of antibiotics on the metabolic integrity of the organisms. Determination of D-lactic acid production may be an additional means of assessing the effects of antimicrobial agents in many pathogenic bacterial species. Logically, the immediate arrest of metabolic activity in microorganisms during the early phase of therapy should be important in halting further tissue damage and further complications resulting from microbial invasion. The importance of these factors could perhaps be examined by further studies by using D-lactic acid production as a probe to correlate in vitro antibiotic test results with results from human and experimental animal infection experiments.

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