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Relation of Beta-Lactamase Activity to Antimicrobial Susceptibility in *Serratia marcescens*

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One-hundred clinical isolates of *Serratia marcescens* were tested for susceptibility to cephalothin, carbenicillin, ticarcillin, ampicillin, and cefoxitin. The majority of the 100 isolates (≥70%) were susceptible to carbenicillin, ticarcillin, and cefoxitin; less than one-half were susceptible to ampicillin; none were susceptible to cephalothin. Ten isolates from the 100 organisms tested were selectively assayed for their β-lactamase activity. Enzyme activity was measured using either iodometric or spectrophotometric methods, and the microbiological assay technique. It was concluded that β-lactamase production was not the sole determinant in β-lactam antibiotic resistance. Resistance without demonstrable β-lactamase was evident in strains for cephalothin, ampicillin, and cefoxitin. In addition, one strain which was susceptible to all antibiotics except cephalothin, elaborated considerable β-lactamase activity.

Infections with *Serratia* have become increasingly more common in the hospitalized patient, especially those receiving antimicrobial agents for protracted periods. These infections present a therapeutic challenge because of extraordinary resistance to most antibiotics. Recently, a proposal has been made to revise speciation within the genus *Serratia* (6). Johnson and Ellner (11), in a report on the distribution of *Serratia* species in clinical specimens, demonstrated the majority (87%) of isolates recovered were *S. marcescens*. Due to variable susceptibility patterns which have been reported for this organism (11) it was considered of interest to correlate elaboration of β-lactamases with susceptibility to β-lactam antibiotic. There is suggestive evidence which might link some of the up-surge in *Serratia* infections with widespread use of certain β-lactam antibiotics (17). In general, production of β-lactamase infers resistance to enzyme-lable antibiotics (10). However, it has been shown (15) that levels of resistance to various β-lactam antibiotics often do not correlate with rates at which these antibiotics are hydrolyzed by β-lactamase.

One-hundred clinical isolates of *S. marcescens* were tested for susceptibility to cephalothin, carbenicillin, ampicillin, and cefoxitin. Cefoxitin is a new cephalosporin-like antibiotic (cephamycin) reported to be highly resistant to β-lactamases from gram-positive and gram-negative bacteria (18). Ten isolates were then selected for enzyme analysis based on their representative susceptibility patterns. These isolates were assayed for their ability to produce β-lactamases capable of degrading these antibiotics in an attempt to determine if differences in resistance profiles could be correlated with β-lactamase elaboration.

MATERIALS AND METHODS

Cefoxitin was generously supplied by Merck Sharp and Dohme Research Laboratories. Ticarcillin was a gift from Beecham-Massengill Pharmaceuticals; ampicillin from Wyeth Laboratories; and cephalothin from Eli Lilly and Co. A commercial preparation of carbenicillin was used.

Cultures. The *S. marcescens* isolates used in this study were obtained from a variety of clinical specimens, submitted to departments of Bacteriology, Mayo Clinic, Rochester and University of Illinois Hospital, Chicago. Two nonclinical isolates, Bizio and 08, have been described elsewhere (16). Stock cultures were maintained on tryptic soy agar slants at 4 C. Determinations of minimal inhibitory concentrations (MIC) were made using a serial twofold broth dilution method in tryptic soy broth (12). Six-hour cultures were diluted 1:1,000 before addition of 0.05 ml (10^3 cells) to the twofold antibiotic dilution. The antibiotic concentrations tested ranged from 100 to 0.09 μg/ml except when MICs were >1,000 μg/ml, in which case the concentration range was 10,000 to 1.95 μg/ml.

Assay for β-lactamase production. Assays for β-lactamase activities were conducted with penicillin G, ampicillin, and carbenicillin, as well as with cephalothin and cefoxitin as substrates. One gram of wet packed cells of an isolate was suspended in 10 ml of 0.2 M potassium phosphate buffer, pH 6.5, and

sonicated for 5 min at 20 kc/s with ice-water cooling. Cell debris was removed from the suspension by centrifugation at 12,000 × *g* for 20 min. The resulting supernatant fluid was assayed for β-lactamase activity. When penicillin G, ampicillin, and carbenicillin were used as substrate, the iodometric method of Perret was used (19). The substrate solution in a 0.2 M phosphate buffer, pH 6.5, was shaken along with the enzyme preparation (0.1 ml) at 30 C for 1 h. The enzyme reaction was stopped by the addition of an iodine reagent, which consisted of 0.0166 N iodine and 0.06 M potassium iodine in a 1.75 M sodium acetate buffer, pH 4. At the end of 10 min the mixture was titrated with 0.0166 N thiosulfate. Under these conditions the consumption of 1 ml of 0.0166 N iodine is equivalent to 2 μmol of penicillin G destroyed (19). The enzyme activity in this case was calculated as micromoles of penicillin G destroyed in 0.1 ml of supernatant fluid per min.

When cephalothin and cefoxitin were used as substrate, the method of Onishi et al. was used (18). Cells of the bacterial isolates tested were suspended in 0.067 M potassium phosphate buffer, pH 7.0, and diluted to a standard optical density (0.10) at a wavelength of 550 nm. The antibiotic solutions containing the cell suspensions were incubated for 1 h at 37 C and the hydrolyzed antibiotic was determined. A starch-iodine indicator in 0.5 M actate buffer, pH 4.0, was added to the enzyme reaction mixture. Exactly 10 min after mixing the starch-iodine indicator reduction was determined spectrophotometrically at 620 nm. The antibiotic hydrolysis was determined from standard curves. A unit of activity was expressed as micromoles of antibiotic degraded per minute per milligram of protein of extract. Protein was determined by the method of Lowry et al. (13). The destruction of penicillin G by supernatant fluid of the various isolates was also determined by microbiological assay method (14). *Staphylococcus aureus* NRRL B313 was used as the test organism (MIC penicillin G = 0.8 μg/ml). Aliquots from the enzyme reaction mixtures for the iodometric assays were taken and diluted 100-fold with iodine reagent to stop the reaction. Residual penicillin G was determined by the agar diffusion method and compared to standards containing an enzyme reaction mixture devoid of the enzyme. Zones of inhibition sizes were measured against a standard solution of the β-lactam substrate. Reductions in the size of the inhibition zones indicated a reduction in the β-lactam substrate concentrations that were a result of enzymatic breakdown of the antibiotic. The concentration of iodine used did not have any inhibitory effect of *S. aureus* growth or antibiotic activity.

RESULTS

Broth dilution tests. Test results for in vitro susceptibility of the 10 selected isolates of *S. marcescens* are presented in Table 1. Two strains, MM Lab and 13378, were resistant to the five β-lactam antibiotics tested. All cultures tested were found resistant to cephalothin at high levels; 3910 and 2736, however, demonstrated significantly lower levels of resistance. Several strains were resistant to ampicillin but susceptible to carbenicillin. Three of the 10 isolates were not susceptible to cefoxitin. In general, the susceptibility patterns of the 10 selected isolates reflected those of the 100 isolates originally tested. Comparable susceptibility was observed for carbenicillin, ticarcillin, and cefoxitin; intermediate susceptibility to ampicillin; none were inhibited by cefalothin (Table 2).

Substrate profiles. Three isolates, MM Lab, 13378, and 2736, were found to elaborate β-lactamases capable of utilizing the five β-lactam compounds as substrates (Table 3). Activities for each substrate are presented as a per-

TABLE 1. *MIC of 10 selected isolates of pigmented and nonpigmented Serratia marcescens*

Source	Antibiotic					Organism
	AM[a]	CB	TC	KF	CX	
Nonpigmented						
NC[b]	3.12[c]	1.56	1.56	10,000	125	Bizio
BL	25.0	0.78	0.78	10,000	15.6	6292
BL	12.5	6.25	6.25	1,250	62.5	3910
Pigmented						
NC	12.5	12.5	12.5	10,000	15.6	08
OR	12.5	12.5	12.5	10,000	7.8	16344
U	>100.0	100.0	1,000	10,000	250	MM Lab
U	>100.0	100.0	250	10,000	250	13378
U	6.25	3.12	3.12	625	7.8	2736
SP	>100.0	1.56	1.56	10,000	15.6	4542
SP	>100.0	1.56	1.56	10,000	15.6	6682

[a] AM, Ampicillin; CB, carbenicillin; TC, Ticarcillin; KF, cephalothin; CX, cefoxitin.
[b] NC, Nonclinical; BL, blood, OR, oral incision; U, urine; SP, sputum.
[c] Concentration in micrograms per milliliter.

TABLE 2. *Susceptibility of 100 isolates of Serratia marcescens to five β-lactam antibiotics*

Antibiotic	Percentage of strains in each MIC category							
	≤1.56[a]	3.12	6.25	12.5	25.0	50.0	100	>100
AM[b]		4	4	38	15	20	4	15
CB	53	20	9	9				9
TC	56	16	9	9				9
KF								100
CX		6	23	41	19	5		6

[a] Concentration in micrograms per milliliter.
[b] For abbreviations see Table 1.

TABLE 3. *Enzyme activities (β-lactamase) elaborated by selected Serratia marcescens isolates for several β-lactam compounds*

Organism	β-Lactamase activities[a]				
	Penicillin G	Ampicillin	Carbenicillin	Cefoxitin	Cepholothin
Bizio	1	0	0	0	0
6292	0	0	0	0	0
3910	0	0	0	0	0
08	0	0	0	0	0
16344	0	0	0	0	0
MM Lab	94	100	93	100	94
13378	85	95	58	97	100
2736	100	99	100	40	72
6682	0	0	0	0	0
4542	0	0	0	0	0

[a] Activities for each substrate are presented as a percentage of the highest activity observed.

centage of the highest activity observed with the 100% values for each substrate. The hydrolysis of the five β-lactam antibiotics by MM Lab and 13378 correlated with the observed MICs. (Table 1). It was of interest, however, and unexpected, that strain 2736 was found to elaborate β-lactamase activity but was susceptible to ampicillin, carbenicillin, ticarcillin, and cefoxitin. Of the 10 isolates, strain 2736 was also the most susceptible to cephalothin (Table 1). In addition, strains 4542 and 6682, which were resistant to ampicillin, did not elaborate β-lactamases capable of utilizing that antibiotic as a substrate. Cephalothin was not hydrolyzed by the remaining strains even though the majority were not susceptible even at levels of 10,000 μg/ml.

Both the microbiological and iodometric β-lactamase assays for the β-lactam substrates demonstrated identical activity patterns for each organism tested (Table 4). The isolates MM Lab, 13378, and 2736 produced the highest activity in both assay systems; the other isolates exhibited low activities in comparison. The β-lactamase activities exhibited with cephalothin and cefoxitin as substrates were at levels representing the lower limits of the spectrophotometric assay method. Attempts to induce further β-lactamase activity for these substrates by growing the organisms in media containing low levels of the antibiotics were unsuccessful.

DISCUSSION

In the past several years, there has been much work conducted to determine the mechanism(s) of resistance of certain bacterial isolates such as *S. marcescens* to antibiotics. Numerous studies have been conducted by researchers on a variety of resistance mechanisms including enzyme inactivation, bacterial cell wall lipids as permeability barriers, and pigmentation. (1, 2, 3, 4, 20) However, there is yet much controversy about what specific factors, or a combination of factors contribute to resistance.

Reports have indicated that nonpigmented strains of *Serratia* are more drug resistant than their pigmented counterparts (5, 9). Although greater drug resistance was demonstrated by pigmented strains in this study of 10 selected isolates, this pattern was not evident when susceptibility for the 100 isolates was examined. Two recent papers (3, 20) have shown there is no correlation between pigment production and antibiotic susceptibility patterns in *S. marcescens*.

TABLE 4. *β-Lactamase activity of selected strains of Serratia marcescens assayed by the iodometric method and microbiological method*

Organism	Iodometric method[a]	Microbiological method[b]
Bizio	1.0	1
6292	0	5
3910	0	2
08	0	6
16344	0	2
MM Lab	95.0	100
13378	85.0	100
2736	100.0	100
6682	0	7
4542	0	2

[a] β-Lactamase activity was expressed in micromoles of penicillin G destroyed per minute per 0.1 ml of extract. The strain with the highest activity was expressed as 100%.

[b] β-Lactamase activity was expressed as zone size of inhibition. The strain with the highest activity was expressed as 100%.

A comparison of subtrate profiles with MICs of the 10 isolates to β-lactam antibiotics indicated that β-lactamase was not the sole mechanism for resistance. This finding was suggested in a preliminary report on cephalosporin resistance in *Serratia* (E. B. Winshell and H. C. Neu, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 12th Atlantic City, N.J., Abstr. 156, p. 82, 1972.) Two isolates (MM Lab, 13378) were resistant to the five β-lactam antibiotics and elaborated good β-lactamase production. Strains of *Serratia* have been reported susceptible to cefoxitin, in part due to greater stability of this new antibiotic to β-lactamases from gram-negative organisms (18). It would appear these two strains elaborate a β-lactamase(s) with broad hydrolytic capabilities including cefoxitin. A third strain, 2736, also elaborated comparable β-lactamase activity, however, this strain was susceptible to all antibiotics except cephalothin. Additionally, the resistance of two isolates to ampicillin did not indicate the production of a β-lactamase capable of utilizing that antibiotic as a substrate.

Neu (17) reported strains of *S. marcescens* resistant to cefoxitin which failed to hydrolyze this antibiotic. One of the 10 isolates (Bizio) in this study was resistant to cefoxitin, however, no β-lactamase activity was demonstrated. This finding confirms that reported by Neu. Neu has further reported that strains of *S. marcescens* which are susceptible to cefoxitin tend also to be susceptible to carbenicillin and ticarcillin (BRL 2288). With the exception of strain Bizio, the susceptibility patterns of the 100 isolates studied would support Neu's finding.

Resistance without demonstrable β-lactamase activity was evident in all strains with cephalothin and two strains with ampicillin. A basic tolerance is reported to be an important determinant in the response of a bacterial strain to an antibiotic(s) (7, 8, 18). In these instances where β-lactamase activity was not demonstrated, basic tolerance could be the primary factor responsible for drug resistance. The findings of this study further support and demonstrate that β-lactamase may play a role in β-lactam antibiotic resistance, but it is not the sole determinant in strains of *S. marcescens*. Other factors such as surface lipid content and the barrier functions of lipopolysaccharide in the outer-membrane of the cell envelope should also be considered.

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