

Increased Resistance of *Pseudomonas aeruginosa* to Carbenicillin After Reversion from Spheroplast to Rod Form¹

CHATRCHAI WATANAKUNAKORN, JOHN P. PHAIR, AND MORTON HAMBURGER

Infectious Disease Division, Department of Medicine, University of Cincinnati Medical Center and the Cincinnati General Hospital, Cincinnati, Ohio 45229

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Five of six strains of *Pseudomonas aeruginosa* had at least fourfold increase in resistance to carbenicillin after they reverted from carbenicillin-induced spheroplasts to rod forms. The most susceptible laboratory strain showed the greatest increase in resistance. There was a marked inoculum effect on the minimal inhibitory concentration of carbenicillin when high inocula (10^7 colony-forming units) or very low inocula (10^2 or 10^3 colony-forming units) were used. Population analysis of this reverted strain revealed increase in resistance of the entire population of cells to carbenicillin. However, the degree of resistance was not homogenous. Only a few cells were highly resistant. Comparison of the effect of carbenicillin on the growth of the parent and reverted strains confirmed the increase in resistance of the reverted strain. The resistance remained stable after 42 passages.

Carbenicillin (disodium alpha-carboxybenzyl penicillin) is a recently developed semisynthetic penicillin which has moderate activity against *Pseudomonas aeruginosa* (1, 11). Because of its lack of toxicity, carbenicillin has been used extensively, with promising results, in England, Australia, and in clinical trials in the United States in the treatment of infections caused by *P. aeruginosa* (3-5, 14, 19). However, there are reports of increased resistance to carbenicillin of *P. aeruginosa* isolated from patients treated with this antibiotic (10, 15, 17-19). Increase in the proportion of carbenicillin-resistant strains of *P. aeruginosa* isolated from patients has also been recently reported from England (12). Many factors may be involved in this increase in resistance. It is the purpose of this communication to report a means by which increase in resistance of *P. aeruginosa* to carbenicillin developed.

MATERIALS AND METHODS

***P. aeruginosa*.** A very susceptible laboratory strain of *P. aeruginosa* (Be) was converted into spheroplasts in hypertonic broth with 100 μ g of carbenicillin (Be) per ml by a method previously described (21). Five clinical strains of *P. aeruginosa* (He, De, Mo, Sy, and Ba) were similarly converted into spheroplasts with 6,000 μ g of carbenicillin per ml.

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All strains of spheroplasts reverted to rod forms within 10 days in the presence of carbenicillin. After reversion, they were maintained on Trypticase Soy Agar (TSA) slants in the absence of carbenicillin.

Carbenicillin. Carbenicillin as a disodium salt was supplied by Beecham Pharmaceuticals, Clifton, N.J. It was dissolved in phosphate buffer (pH 6) and stored at 4 C.

Susceptibility testing methods. A serial twofold broth dilution method previously described (13) was used to determine the minimal inhibitory concentration (MIC) of carbenicillin. The MIC values were recorded after 18 and 36 hr of incubation at 37 C. To determine the effect of inoculum size of strain Be and its reverted strain (R Be) on the MIC, inocula of 10^2 to 10^7 cells were used.

Population analyses of strains Be and R Be. A 0.1-ml amount of tenfold dilutions of an 18-hr culture of strain Be or R Be was inoculated onto the surface of TSA plates with and without carbenicillin. Colonies were counted after 5 days of incubation at 37 C.

Effect of carbenicillin on the growth of strains Be and R Be. Carbenicillin was added to an appropriate dilution of an 18-hr culture of strain Be or R Be in Trypticase Soy Broth (TSB) to make the desired final concentration. A similar broth culture of the same strain of *P. aeruginosa* without carbenicillin was set up as a control. The cultures were incubated at 37 C. Colony counts for viable colony-forming units (CFU) before and at intervals after *P. aeruginosa* was exposed to carbenicillin were determined by the pour-plate technique using TSA. Ten thousand units of *Bacillus cereus* penicillinase (Neutrapen, Riker Laboratories, Northridge, Calif.) was added to each TSA

plate to inactivate carbenicillin present in the inoculum.

RESULTS

Susceptibility of parent and reverted strains of *P. aeruginosa* to carbenicillin. Table 1 lists the MIC of carbenicillin for six strains of *P. aeruginosa* and their respective reverted strains when 10^6 to 3×10^6 CFU were used as the inoculum. Five of the six reverted strains exhibited at least fourfold increase in MIC. Strain Be, which was the most susceptible strain, showed the greatest increase in resistance after reversion. The MIC increased from 2 to 500 $\mu\text{g/ml}$ (18-hr reading) and from 3.9 to 2,000 $\mu\text{g/ml}$ (36-hr reading).

Inoculum effect on the MIC. Table 2 shows the effect of inoculum size on the MIC of carbenicillin for strain Be and its reverted strain R Be. Very high inocula (10^7 CFU) and very low inocula (10^2 and 10^3 CFU) markedly affected the MIC of carbenicillin for both strains. There was no significant difference between the MIC values obtained when 10^4 , 10^5 , or 10^6 CFU were used as inocula. The values of MIC of carbenicillin for

the reverted strain were greatly increased, regardless of the size of the inoculum.

Population analysis. Tables 3 and 4 demonstrate the heterogenous nature of the susceptibility of the cell population of both the parent Be and the reverted R Be strains to carbenicillin. Only 3 of the 10^7 cells of the parent strain were resistant to 25 μg carbenicillin per ml, and none was resistant to a concentration of 50 $\mu\text{g/ml}$. The cell population of the reverted strain showed a marked increase in resistance, although the number of highly resistant cells was small. Thus, 6 of the 10^7 cells were resistant to 1,500 $\mu\text{g/ml}$. With smaller inocula, fewer highly resistant cells were found. None of the 10^5 inoculum was resistant to 500 $\mu\text{g/ml}$, and only 13 were resistant to 250 $\mu\text{g/ml}$. When 200 cells were studied, 4 of them grew in a concentration of 75 $\mu\text{g/ml}$. Therefore, regardless of the size of population of cells studied, the reverted strain had marked increase in resistance.

Comparison of the effect of carbenicillin on the growth of the parent and reverted strains of *P. aeruginosa*. Figure 1 compares the effect of 100 μg of carbenicillin per ml on the growth of *P. aeruginosa* strain Be and its reverted strain R Be. The growth rate of both strains in the control cultures was comparable. With carbenicillin, the viable colony count of the parent strain at 24 hr was 4 logs lower than the reverted strain, and the difference was even greater at 48 hr. This again confirmed the marked increase in resistance of the cell population of the reverted strain.

Stability of resistance of the reverted R Be strain. After 42 passages of the reverted R Be strain in TSB without carbenicillin, the MIC of carbenicillin was found to be unchanged. Figure 2 shows the effect of 50 μg of carbenicillin per ml on the growth of *P. aeruginosa* strain Be and its reverted strain R Be after 42 passages. It is obvious that the reverted strain retained its resistance after many passages.

Penicillinase production and pyocine typing. Penicillinase production by both the parent and reverted Be strains of *P. aeruginosa* was tested by the method of Haight and Finland (9) and none was detected. Pyocine typing (6, 8, 20) was performed by Corwin R. Dunn of the Bacteriology Unit, National Communicable Disease Center. The parent and reverted Be strains were found to have the same pyocine type (type 33 of Gilles and Govan, 8).

DISCUSSION

The results of this investigation demonstrate that some strains of *P. aeruginosa* showed a marked increase in resistance to carbenicillin

TABLE 1. Susceptibility of the parent and reverted strains of *P. aeruginosa* to carbenicillin^a

Parent	MIC ^b		Reverted	MIC	
	$\mu\text{g/ml}$			$\mu\text{g/ml}$	
Be	2.0,	3.9	R Be	500.0,	2,000.0
He	39.0,	39.0	R He	312.0,	312.0
De	78.0,	78.0	R De	312.0,	1,250.0
Mo	19.5,	19.5	R Mo	78.0,	156.0
Sy	62.5,	125.0	R Sy	500.0,	500.0
Ba	312.5,	312.5	R Ba	312.5,	312.5

^a Inoculum = 10^6 to 3×10^6 colony-forming units.

^b Minimal inhibitory concentration. First value, 18-hr reading; second value, 36-hr reading.

TABLE 2. Effect of inoculum size on the susceptibility of *P. aeruginosa* strains Be and R Be to carbenicillin

Inoculum	Be MIC ^a		R Be MIC	
	$\mu\text{g/ml}$		$\mu\text{g/ml}$	
$2-3 \times 10^7$	31.2,	31.2	4,000,	8,000
$2-3 \times 10^6$	2.0,	3.9	500,	2,000
$2-3 \times 10^5$	1.0,	2.0	250,	500
$2-3 \times 10^4$	0.5,	1.0	125,	500
$2-3 \times 10^3$	0.125,	0.25	62.5,	250
2×10^2	0.06,	0.25	15.6,	250

^a Minimal inhibitory concentration. First value, 18-hr reading; second value, 36-hr reading.

TABLE 3. Population analysis of *P. aeruginosa* strain Be

Inoculum	Concn of carbenicillin ($\mu\text{g/ml}$) ^a										
	50	25	10	5	2	1	0.5	0.25	0.1	0.05	0
2.0×10^7	0	3	225	314	+	++	++	++	++	++	++
2.0×10^6	0	0	0	0	130	278	+	++	++	++	++
2.0×10^5	0	0	0	0	0	63	+	++	++	++	++
2.0×10^4	0	0	0	0	0	2	11	+	++	++	++
2.0×10^3	0	0	0	0	0	1	0	+	++	++	++
2.0×10^2	0	0	0	0	0	0	3	30	190	200	191
2.0×10^1	0	0	0	0	0	0	0	1	20	18	20

^a Numerals = numbers of colonies, + = discrete colonies but too numerous to count, ++ = confluent growth.

TABLE 4. Population analysis of reverted Be strain of *P. aeruginosa*

Inoculum	Concn of carbenicillin ($\mu\text{g/ml}$) ^a										
	1,500	1,000	750	500	250	100	75	50	25	0	
2.0×10^7	6	8	++	++	++	++	++	++	++	++	
2.0×10^6	0	2	46	+	++	++	++	++	++	++	
2.0×10^5	0	0	0	0	13	303	+	++	++	++	
2.0×10^4	0	0	0	0	0	6	516	++	++	++	
2.0×10^3	0	0	0	0	0	0	59	52	+	+	
2.0×10^2	0	0	0	0	0	0	4	5	32	262	
2.0×10^1	0	0	0	0	0	0	0	0	0	20	

^a Numerals = numbers of colonies, + = discrete colonies but too numerous to count, ++ = confluent growth.

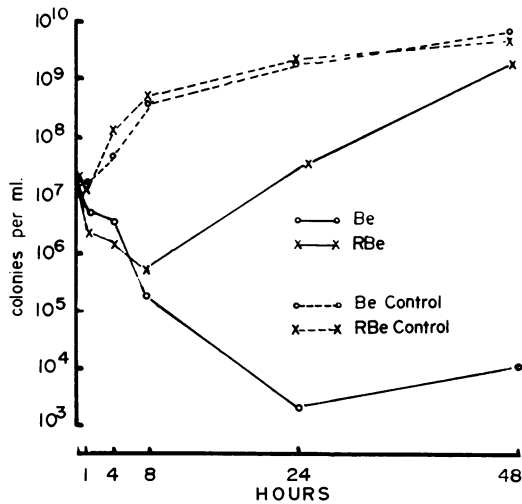


FIG. 1. Comparison of the effect of 100 μg of carbenicillin per ml on the growth of *P. aeruginosa* strain Be and its reverted strain RBe.

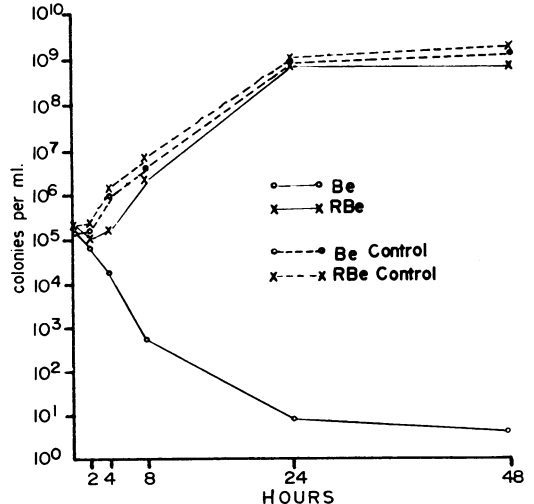


FIG. 2. Comparison of the effect of 50 μg of carbenicillin per ml on the growth of *P. aeruginosa* strain Be and its reverted strain RBe (42nd passage).

after they had been converted into spheroplasts by carbenicillin and reverted back to rod forms. For one strain, studied in detail, the entire population of cells of the reverted strain displayed

marked increase in resistance, though individual cells varied in the degree of resistance. The exact mechanism for this increase in resistance is not known; it was not due to the production of peni-

cillinase, and the resistance was stable after many passages. Since the highly resistant cells in the reverted strain were not present in the population of cells of the original strain, it is unlikely that the increase in resistance represented the result of selective induction of the more resistant cells of the original strain into spheroplasts and subsequent reversion.

Several *in vitro* studies have demonstrated development of resistance of *P. aeruginosa* to carbenicillin after sensitive strains were serially passed in media containing increasing concentrations of the antibiotic (7, 15, 16). Bell and Smith demonstrated the selection of virulent carbenicillin-resistant variants of *P. aeruginosa* by growing heavy inocula on agar plates containing carbenicillin (2). Lowbury and his associates (12) found that highly resistant strains of *P. aeruginosa* isolated from clinical materials produced carbenicillinase. They also found that the resistance was unstable and that exposure to acriflavine enhanced reversion to sensitivity. This prompted them to postulate that an extrachromosomal factor was responsible for the resistance.

The increasing reports of isolation of carbenicillin-resistant strains of *P. aeruginosa* from clinical material may be of therapeutic importance. Though the exact mechanism for this increase in resistance is not clear, any of the above mentioned means by which increased resistance developed *in vitro*, including our own findings, might be operative *in vivo*.

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