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ANTIBIOTIC-RESISTANCE PATTERNS OF SOIL BACTERIA (GRAM-NEGATIVE RODS) FROM THE KRAKATAU ISLANDS (RAKATA) AND WEST JAVA, INDONESIA, IN 1984

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CONTENTS

The explosive eruption of the Indonesian island of Krakatau (Krakatoa) in 1883 almost certainly led to the total destruction of the fauna, flora and microorganisms of the three remaining islands, Rakata (Rakata Besar), Sertung and Panjang (Rakata Kecil). The Ujung Kulon peninsula of Java, 100 km to the south, was much less affected by the volcanic activity.

Soil bacteria (Gram-negative rods, GNR) from Rakata (the post-1883 remnant of Krakatau) and the Ujung Kulon peninsula of Java were examined for their antibiotic-resistance patterns. A total of 27 patterns ('resistotypes') was detected, based on resistance to the antibiotics ampicillin, chloramphenicol, sulphamethoxazole, trimethoprim, gentamicin, cephalothin and tetracycline. Nine resistotypes were common to Rakata and Java, eleven exclusive to Rakata, and seven exclusive to Java. Two of the common resistotypes (f and z) were widely distributed but most were limited in their distribution and detected at only one site. On both Java and Rakata different resistotypes were detected at different altitudes. Effective

colonization of Rakata by GNR has occurred in the 101 years since its sterilization. Seeding of the upper slopes and summit of Rakata with biotypes from mountain areas in Java appears to have occurred. In addition, apparently independent evolution of GNR has occurred on Rakata.

Overall, the GNR detected on the uninhabited and previously sterilized island of Rakata were resistant to as wide a range of antibiotics as were those from nearby Java.

1. Introduction

Over one hundred years have now elapsed since the cataclysmic volcanic eruption of the Indonesian island of Krakatau (Krakatoa) (figures 1 and 2) in 1883. The surface of the island was still hot two months after the eruption, and it is likely that Rakata (the remnant of Krakatau) and the adjacent islands Sertung and Panjang, were sterilized by this volcanic event. The destruction of the fauna, flora (and presumably microbiota) of the islands has provided scientists with a unique opportunity to study the reassembly of a tropical ecosystem from a sterile baseline (Thornton 1984). Apart from a few reports (Treub 1888; Ernst 1908; van Baren 1931; Feher 1936), microbiologists have not been involved in this long-term investigation and the present study is an attempt to rectify this omission. The aim was to compare the antibiotic resistance of soil Gram-negative rods (GNR) on Rakata with an area of Java 100 km to the south (figure 1) where before 1883 some shifting cultivation occurred, and which is now an Indonesian national park. Specifically, we wished to ascertain whether the soil bacteria of an inhabited land mass, where antibiotics are extensively used, differ in their antibiotic-resistance patterns from those of an uninhabited island thought to have been previously sterilized by volcanic eruption.

The 1984 La Trobe University Zoological Expedition to the Krakatau Islands (Thornton & Rosengren 1988) was an opportunity to investigate this. Soil samples obtained on Java and Rakata were returned to Australia for microbiological examination.

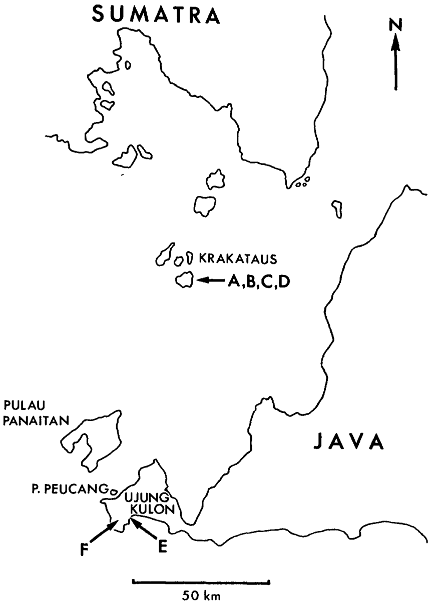

FIGURE 1. Sunda Strait showing position of the Krakatau Islands and Ujung Kulon peninsula, where collecting sites A to F were situated.

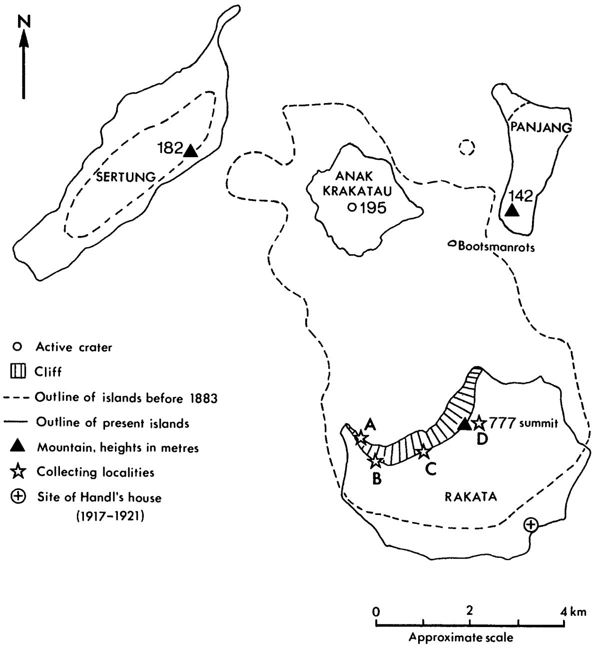

FIGURE 2. The Krakatau Islands, showing collecting sites A to D on Rakata.

Figure 2 shows that the Krakataus consist of four main islands, of which Rakata is the remnant of the original and much larger pre-1883 Krakatau, and it is presumed to have been sterilized by the eruption of 1883. A family lived on the southeast coast (remote from the sites sampled in this study) from 1917 to 1921 (figure 2). There have been no other permanent inhabitants, but Zwarte Hoek (site A, figure 2) is sometimes used as a temporary shore camp by fishermen and smugglers. The island is now covered in mixed secondary forest.

The island of Java is a very much larger land mass to the south and east of the Krakataus (figure 1). Only the Ujung Kulon peninsular was investigated (figure 3) in 1984. Inhabited up

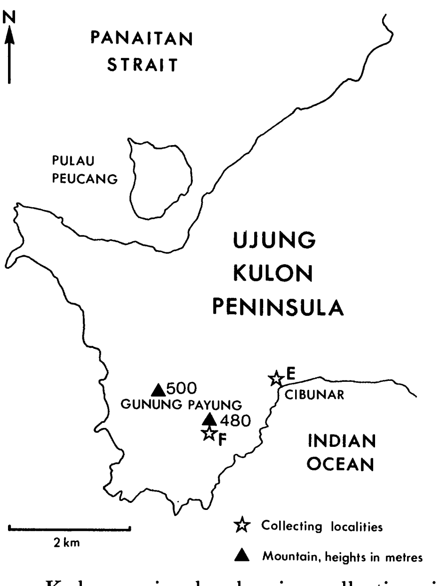

FIGURE 3. Ujung Kulon peninsula, showing collecting sites E and F.

to 1883, it is now a wilderness area and wildlife reserve for the Javan rhinoceros, but there is a permanent guard post on Pulau Peucang, temporary shelters for guard patrols (one at Cibunar), and sporadic human traffic in the form of tourists, scientists and a few poachers.

In 1985 the La Trobe University/L.I.P.I. Zoological Expedition to the Krakatau Islands extended this survey to the islands of Sertung and Anak Krakatau, and the results of this work will be reported upon in a later paper (Graves *et al.* 1988).

2. Materials and methods

(*a*) *Soil samples*

The expedition took place in August–September 1984. Soil samples were collected aseptically at six different locations on Rakata and Java (tables 2 and 3, figures 1–3). Soil samples were taken at 5 cm below the litter layer, at sites well away from trails and from existing or possible previous campsites. Brief descriptions of the six collecting sites follow (see figures 1–3).

Rakata

Site A. Zwarte Hoek, 50 m inland from beach, among rocks.

Site B. Western ridge, 300 m altitude, 7 m south of cliff edge, among ferns and tree roots.

Site C. Western ridge, 500 m altitude, 3 m south of cliff edge, among thick vegetation.

Site D. Summit area (777 m), 3 m south of cliff edge, among tree roots.

Java (*Ujung Kulon peninsula*)

Site E. Cibunar stream, 25 m inland from mouth and 10 m east of stream (6 m east of temporary guard patrol shelter), among very dense vegetation.

Site F. Gunung Payung, summit (480 m), west of summit marker, in dense vegetation.

Sterile plastic screwtop containers with a sterile spatula attached to the inside of the lid were used for collecting soil samples. At each site, four separate samples were collected, to give a total of 24. The samples were flown back to Melbourne, Australia, but could not be refrigerated at any stage before microbiological analysis. Because of the logistics of the expedition and administrative difficulties in having the samples admitted to Australia (which has extremely stringent quarantine regulations concerning the importation of living biological material), the soil did not undergo microbiological examination until approximately five weeks after collection.

(*b*) *Culture procedures*

A 0.2 g sample of each soil specimen was added to 10 ml of sterile NaCl (9 g l^{-1}) and well suspended with a vortex mixer. With the use of a calibrated inoculating loop, 0.01 ml of the sample was lawn plated on to Horse Blood Agar (HBA) and MacConkey Agar (MCA) and incubated at 37 °C for 2 days.

Colonies on both plates were counted and the appropriate adjustment made to determine the number of colony-forming units per gram of soil. One colony per plate represented 5×10^3 bacteria per gram of soil (the lowest concentration detectable in this study).

Colonies on the MCA plate were Gram-stained to determine which were GNR, and the MCA plate was then replica-plated on to seven separate antibiotic-containing agar plates. Each plate contained one of the following: ampicillin 8 μg ml^{-1}, chloramphenicol

8 μg ml^{-1}, sulphamethoxazole 20 μg ml^{-1}, trimethoprim 1 μg ml^{-1}, gentamicin 4 μg ml, cephalothin 8 μg ml^{-1}, tetracycline 4 μg ml^{-1}.

The appearance or absence of the original colonies on the antibiotic plates was recorded and the pattern of antibiotic resistance (termed 'resistotype', after Routman *et al.* (1985)) of the GNR was determined (table 1) depending on resistance or sensitivity to the range of antibiotics at the concentration in the agar.

Only rarely was it possible to determine the genus of the bacterium isolated.

Table 1. Resistotypes of GNR as defined by their antibiotic-resistance patterns

	antibiotic to which the resistotype is resistant (R) or sensitive (S)							number of antibiotics to which resistotype is resistant
resistotype	ampicillin 8 μg ml^{-1}	chloramphenicol 8 μg ml^{-1}	sulphamethoxazole 20 μg ml^{-1}	trimethoprim 1 μg ml^{-1}	gentamicin 4 μg ml^{-1}	cephalothin 8 μg ml^{-1}	tetracycline 4 μg ml^{-1}	
a	R	R	R	R	R	R	R	7
b	R	R	R	R	R	R	S	
c	R	R	R	R	S	R	R	6
d	R	R	R	S	R	R	R	
e	R	R	S	R	R	R	R	
f	R	R	R	R	S	R	S	
g	R	S	R	R	R	R	S	
h	R	S	R	R	S	R	R	5
i	R	S	R	S	R	R	R	
j	R	S	S	R	R	R	R	
k	S	S	R	R	R	R	R	
l	R	R	R	R	S	S	S	
m	R	S	R	R	S	R	S	4
n	R	S	R	R	S	S	R	
o	R	R	S	R	S	S	S	
p	R	S	R	R	S	S	S	
q	R	S	S	R	R	S	S	3
r	R	S	S	R	S	R	S	
s	S	S	R	R	S	R	S	
t	S	S	S	R	S	R	R	
u	R	S	S	R	S	S	S	
v	S	S	R	R	S	S	S	2
w	S	S	S	R	R	S	S	
x	S	S	S	R	S	R	S	
y	S	S	R	S	S	S	S	1
yy	S	S	S	R	S	S	S	
z	S	S	S	S	S	S	S	0

3. Results

(a) *Resistotypes of* GNR

Twenty-seven different patterns of antibiotic resistance were detected (table 1). They ranged from 'a' (resistant to all seven antibiotics) to 'z' (sensitive to all). Two resistotypes were very frequent (figure 4); 'f' (resistant to all antibiotics except gentamicin and tetracycline) and 'z' (sensitive to all antibiotics). Twenty resistotypes were detected on Rakata compared with 16 on Java. Of the 27 resistotypes detected, only nine were common to both areas (a, b, c, e,

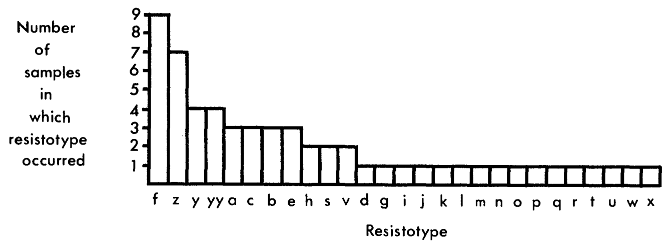

FIGURE 4. Resistotype frequencies of GNR detected at all sites on Rakata and Java.

f, h, v, yy, z). Eleven were exclusive to Rakata (i, j, k, l, q, r, t, u, w, x, y) and seven exclusive to Java (d, g, m, n, o, p, s).

The variation in resistotype as a function of elevation, on both Java and Rakata, is noteworthy. On Java completely different resistotypes were detected at Cibunar (sea level, at the foot of Gunung Payung) and at the summit of the mountain (480 m) (table 2). On Rakata, of the eleven resistotypes detected at the summit of the island seven were not detected at any of the lower sites (e, j, k, q, r, u, x). The significance of this is not clear, although it is probably a reflection of different vegetation at various altitudes.

TABLE 2. ANTIBIOTIC-RESISTANCE PATTERNS (RESISTOTYPES) OF SOIL BACTERIA (GNR) ON RAKATA AND JAVA

	site	sample no.	resistotypes detected in sample	resistotypes detected at site
Rakata	A. beach at Zwarte Hoek	1	no GNR detected	
		2	no GNR detected	
		3	i, z	f, i, z
		4	f	
	B. 300 m elevation	5	f	
		6	c, f	
		7	v, y, z	b, c, f, h, l, v, w, y, z
		8	b, f, h, l, w, z	
	C. 500 m elevation	9	y	
		10	c, f, t, z	
		11	f, y, yy, z	a, c, f, t, y, yy, z
		12	a, y	
	D. 777 m elevation (summit)	13	a, r, x	
		14	e, f, k, j	
		15	no GNR detected	a, b, e, f, k, j, q, r, u, x, z
		16	b, e, q, u, z	
Java	E. Cibunar (sea level)	17	f, h, m, s, v, yy	
		18	b, d, n, s, yy	
		19	no GNR detected	b, d, f, g, h, m, n, s, v, yy, z
		20	f, g, yy, z	
	F. Gunung Payung (400 m elevation)	21	no GNR detected	
		22	no GNR detected	
		23	a, c, e, o	a, c, e, o, p
		24	p	

It is apparent from figure 5 that both resistant and sensitive resistotypes are present on Rakata and Java. In general, resistotypes on Java and Rakata do not appear to differ in the range of their antibiotic resistance. Moreover, slightly more resistotypes (19) were isolated from the uninhabited island of Rakata than from the Ujung Kulon peninsular (16). On Rakata there is a trend towards more-resistant GNR at higher altitudes.

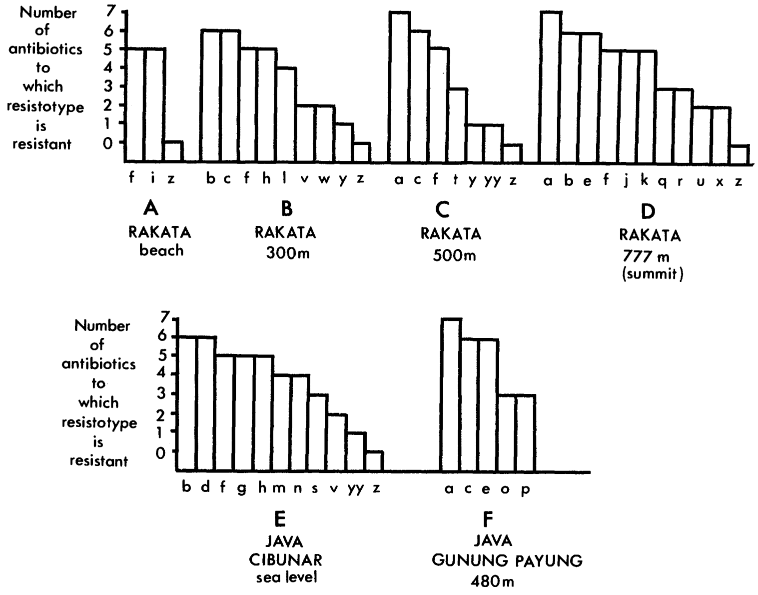

FIGURE 5. Number of antibiotics to which resistotypes of GNR were resistant (maximum 7), by sample site.

TABLE 3. RELATIVE CONCENTRATIONS OF SOIL BACTERIA (GROWTH ON HORSE BLOOD AGAR, 37 °C) ON RAKATA AND JAVA

	site	sample no.	10^{-5} × bacterial concentration per gram soil: each sample	average for site	average for island
Rakata	A. beach at Zwarte Hoek	1	2.0		
		2	20.0	18.0	
		3	10.0		
		4	40.0		
	B. 300 m elevation	5	4.0		
		6	12.0	8.0	
		7	10.0		
		8	6.0		
	C. 500 m elevation	9	6.5		9.9
		10	7.0	8.0	
		11	18.0		
		12	0.4		
	D. 777 m elevation (summit)	13	12.5		
		14	2.5	5.5	
		15	1.2		
		16	6.0		
Java	E. Cibunar (sea level)	17	4.0		
		18	11.0	6.2	
		19	8.0		
		20	2.0		10.1
	F. Gunung Payung (400 m elevation)	21	0.4		
		22	4.5	14.0	
		23	50.0		
		24	1.0		

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(*b*) *Relative concentrations of soil microorganisms*

These figures are approximations only, because during the five weeks between collection of samples and microbiological analysis, the soil was at ambient temperature, although within sterile sealed containers preventing contamination or desiccation.

Overall, there was no difference between Rakata (9.9×10^5 bacteria per gram soil) and Java 10.1×10^5 bacteria per gram soil) (table 3). There was a maximum 100-fold variation, however, from sample to sample (samples 21 and 23), but only a maximum threefold variation from site to site (sites D and A). These data suggest that Rakata soil is now as fully colonized bacteriologically as is Javan soil.

4. Discussion

Earlier studies on soil from the Krakatau Islands by De Kruyff in 1906 (reported by Ernst (1908)), only 23 years after the major eruption, and by Schiutemaker in 1928 (van Baren 1931; Jenny 1941), indicated that the Krakatau soil contained a normal concentration of bacteria. Jenny (p. 36) wrote 'the microbiological population was determined by Schiutemaker and was found to compare favorably with that of a garden soil', whereas Ernst (p. 52) wrote 'the bacteriological investigations...demonstrated the occurrence of between 1.3 and 2.8×10^6) (av: 2.2×10^6) bacteria in 1 gram of soil, figures which agree with those obtained by De Kruyff from an examination of the soil at Buitenzorg (Bogor) in Java...'. It is therefore not surprising that we found no difference in bacterial concentration of soil microorganisms between Rakata and the Ujung Kulon peninsula of Java; bacterial concentrations in the soil of Rakata would have returned to normal many decades ago, probably as soon as vegetation became reestablished (Graves *et al.* 1988). Our values of 10^6 bacteria per gram (table 3) for Java and Rakata soil is close to that obtained by De Kruyff in 1906.

With regard to the antibiotic-resistance patterns of the soil GNR, both Rakata and Java have a variety of resistotypes, and nine were common to both areas. This suggests that during the past 101 years some of the resistotypes from Java have colonized Rakata, probably by wind, plant seeds or flying animals. It is probable that the resistotypes found on Java on the summit of Gunung Payung (480 m) and also at 500 m and on the summit (777 m) of Rakata, are the result of direct spread (resistotypes 'a', 'c', 'e'). This is particularly likely in the case of resistotype 'e', which was present in sample 23 from the summit of Gunung Payung (table 2) at much higher concentrations (3.85×10^5 per gram of soil) than other resistotypes. It was detected at only one other site, the summit of Rakata, where it was also at a higher concentration (0.35×10^5 per gram of soil) than the other resistotypes detected there. However, the presence of 11 unique resistotypes on Rakata suggests that these bacteria have evolved independently on that island within the past century.

Thus, with respect to both microbial concentration and range of GNR resistotypes, the soil of Rakata is probably fully developed bacteriologically and may well have been so for many decades.

A limited effort only was made to identify and classify the bacteria isolated in this study, as this was not its main objective; in contrast, antibiotic-resistance patterns were considered important. Of the nine bacteria identified, seven were species of *Pseudomonas*, a genus well known to be a soil inhabitant. The others were a *Serratia* species and a *Flavobacterium* species, both well-known bacteria of terrestrial habitats.

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The fact that so many of the bacteria found on fruit and vegetables (and presumably originating from the soil) show a high degree of antibiotic resistance has led to some concern that they may be agents of human disease or the donors of antibiotic-resistant plasmids that can convert human and animal intestinal bacteria (both normal flora and pathogens) into antibiotic-resistant strains (Remington & Schimpff 1981). Certainly the exposure of microorganisms to antibiotics does lead to the development of antibiotic-resistant strains, both *in vitro* and *in vivo* (in humans and animals). But is the widespread occurrence of antibiotic-resistant GNR on vegetables and in soil a result of human contamination of the environment with antibiotics (via human and veterinary medicine), or are they present naturally? This is difficult to answer as humans and their domestic and commensal animals are present virtually everywhere, and so are antibiotics. In Antarctica, of 155 strains of bacteria isolated, 48 carried plasmids, and of these, seven showed antibiotic resistance (Kobori *et al.* 1984); only two of the 107 bacteria not containing plasmids were antibiotic resistant. Kobori and his colleagues concluded that bacterial plasmids are ubiquitous in natural microbial populations in Antarctica, where human modification of the environment may be considered minimal. Baya *et al.* (1986) detected antibiotic resistance in marine bacteria from the open sea of the Atlantic Ocean, although isolates from close to sewage treatment plants were more antibiotic resistant.

Rakata was selected for this study because it is believed to have been sterilized by the eruption of 1883, and since that time has been subjected to very limited human contact. There are no permanent human settlements, and no domestic animals or agriculture. Only fishermen, tourists and scientists visit the island, for limited, temporary stays. The environment is not exposed to the normal levels of human and domestic animal faecal pollution. Yet antibiotic-resistant GNR are present in the soil in quite large numbers, and some are resistant to as many as seven antibiotics. The site on Rakata most visited by humans is the beach at Zwarte Hoek, whereas the ridge above the massive cliff (300 m to the 777 m summit) is very rarely visited. However, most of the antibiotic-resistant GNR were detected at the elevated sites and fewer were detected on the beach (figure 5 and table 2). This further suggests that antibiotic-resistant GNR are a part of the normal microbiota of the soil.

From this limited study it is suggested that the occurrence of antibiotic resistance in soil bacteria, and possibly in other terrestrial bacteria, may be the result of natural selection within the natural ecosystem, and may not necessarily be due to the activities of humans.

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