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RESTRICTION ENDONUCLEASE ANALYSIS OF THE DNA OF *RICKETTSIA PROWAZEKII* VACCINE STRAIN E AND ITS REVERTANT[1]

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Summary. — The DNA of *Rickettsia prowazekii* vaccine strain E was analysed by restriction analysis with 17 endonucleases in comparison with its virulent revertant — Evir and the virulent reference strain Breinl. The DNA of cloned and uncloned strains showed identical restriction endonuclease patterns. In spite of stable differences in virulence, strains E and Evir displayed a totally identical DNA cleavage pattern indicating the absence of marked structural differences between their genomes. On the other hand 9 endonucleases showed differences in the restrictograms of the DNA strain Breinl as compared with strains E and Evir.

Key words: *variability*; *Rickettsia prowazekii*; *weakly pathogenic strain* E; *virulent revertant strain Evir*; *restriction endonuclease analysis of DNA*

Introduction

Low virulent strain E of *Rickettsia prowazekii* used for vaccination is a batch empirically selected in the course of chick embryos (CE) passages of the virulent strain (Clavero and Perez-Gallardo, 1944; Perez-Gallardo, 1963). The attenuation of stran E is known to be unstable and dependent on the cultivation conditions. Low pathogenicity of strain E is preserved in the course of passages in CE. The passage of strain E in weakly susceptible albino mice (intranasal inoculation) and guinea pigs (intraperitoneal inoculation), leads to an irreversible increase of its virulence (Balaeva, 1969; Balaeva and Nikolskaya, 1973*a*,*b*; Kazár *et al.*, 1973). This rise in virulence occurred in human amnion cells (Ignatovich, 1975; Balaeva *et al.*, 1976), as well as upon successive passages in clothes lice and then in CE (Balaeva *et al.*, 1976). The mechanism of attenuation and reversion of the virulence of Rickettsia strain E is unclear. It should be noted that strain E rickettsiae are similar to the virulent strain with respect to the toxic and haemolytic activities, the presence of a capsule and cell penetration characteristics

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(Pshenichnov *et al.*, 1976; Perez-Gallardo and Fox, 1948; Silverman and Wisseman, 1978; Winkler and Miller, 1980). The differences between them are in the inability of strain E to multiply in the cytoplasm of phagocytic cells (Gambrill and Wisseman, 1973; Turco and Winkler, 1982; Winkler and Daugherty, 1983). Differences were described in electrophoretic mobility of some proteins and in the DNA cleavage pattern by endonucleases (Oaks *et al.*, 1981; Regnery *et al.*, 1983, 1985).

The purpose of the present paper was to compare the genome of isogenic strains: the weakly pathogenic strain E and its virulent revertant strain Evir, produced by passaging in the lungs of albino mice. The total DNA has been investigated by restriction endonucleases with simultaneous analysis of the DNA of reference virulent strain Breinl of *Rickettsia prowazekii*.

Materials and Methods

Rickettsiae. Three strains of *Rickettsia prowazekii* have been studied: E, Evir and Breinl. Both standard passage variants of the strains and cloned variants were used. The cloning was carried out by plaque method and by limiting dilutions method in CE. Characteristics of the strains are

Table 1. The Rickettsia prowazekii strains

Strain	Passage history	
	Uncloned culture	Cloned culture
E	Isolated by Clavero and Perez-Gallardo (1944) Obtained from Prof. Wisseman (U.S.A.) in 1969; underwent 270 passages in CE	
	282—285 passages in CE	278 passages in CE + 1 passage in tissue culture (cloning by plaque method) + 6 passages in CE
Evir	Prepared by 13 passages in albino mice lungs from strain E (obtained from the U.S.A. in 1969) by Balaeva (1971)	
	7 passages in CE	13 passages in CE + 3 passages in CE in limiting dilutions (cloning) + 1 passage in CE
Breinl	Isolated by Wolbach *et al.* (1922); passaged in guinea pigs (passage history unknown)	
	134 passages in CE	9 passages in CE + 3 passages in CE in limiting dilutions (cloning) + 1 passage in CE

given in Table 1. Yolk sacs from recently died CE that were found to have the greatest amount of rickettsiae were collected and kept at −60 °. They were inactivated with 0.1 % formalin at 6 °C for 24 hr (Regnery *et al.*, 1983).

Purification of rickettsiae from CE tissues. The inactivated rickettsiae were purified by differential centrifugation and verografin density gradient (76 % verografin, Spofa, Č.S.S.R.) using a somewhat modified technique of Weiss *et al.* (1975). At the first step K36 was used as solvent (Weiss *et al.*, 1967). In the second step, the rickettsial suspension was layered over sucrose verografin solution in K36 (30 % sucrose and 7.6 % verografin) and centrifuged at 22 000 × g for 1 hr. The pellet was resuspended in K36 and recentrifuged. At the third stage, discontinuous verografin gradient (20, 30, 37.5, 39, 45 %) was used. Centrifugation was carried out for 1 hr at 87 000 × g (VAK-25 centrifuge, G.D.R.) bucket-rotor 3312, Beckman tubes or the rotor SW 25.1. The rickettsiae formed two layers at 37.5 and 39 % levels. The upper and lower layers were collected, the verografin was removed by washing in K36 and the centrifugation in verografin gradient was repeated. Then verografin was removed by washing in K36, the latter were resuspended in SSC buffer.

Isolation of rickettsial DNA. Total rickettsial DNA was isolated according to the method of Priefer *et al.* (1984). Purified rickettsial cells were diluted to the density equal to OD 5 at wavelength 440 nm. Proteinase K (Serva, F.R.G.) was then added to a final concentration of 250 μg/ml and 10 % sarcosyl (N-lauroylsarcosine, Sigma, U.S.A.) to at final concenration of 0.5 %. The mixture was incubated for 1 hr at 50 °C and then for 15 hr at 37 °C. The lysate was deproteinized 2 or 3 times with phenol and then 2 times more with a mixture of chloroform and isoamyl alcohol (24 : 1). The DNA was precipitated by addition of 0.54 volume of isopropanol in the presence of 0.3 mol/l sodium acetate at room temperature for 15 min. The DNA pellet was rinsed 3 to 5 times with cooled ethanol (70 %) and dissolved in TE buffer, pH 8 at 4 °C for 18 hr.

Restriction endonuclease digestion and electrophoresis. Restriction analysis was carried out as recommended by Maniatis *et al.* (1982) under standard conditions: 1 unit of enzyme was used to cleavage 1 μg DNA at 37 °C for 2 hr with subsequent inactivation of the enzyme for 10 min at 65 °C.

Table 2. Comparison of the restrictograms of Rickettsia prowazekii strain Breinl and of group E strains (E and Evir)

Restrictase	Number of visually discernible DNA bands			Range of fragment distribution (tnp)	Size of additional fragments (tnp)	
	Breinl	E	Evir		Breinl	E, Evir
BamHI	8	9	9	7—50	—*	9.5
BglI	9	9	9	10—50	20	23
*Cfr*13I	36	35	35	1—15	8	—
*Eco*RI	40	41	41	2—25	—	18
*Hind*III	39	38	38	1—15	9	—
*Msp*I	50	50	50	0.5—15	9	8
*Mva*I	46	45	45	1—20	18	—
*Pst*I	50	51	51	1—30	15	20; 16
*Xho*I	12	13	13	6—50	—	15
*Bcn*I	19	19	19	8—50	—	—
*BspR*I	32	32	32	1—30	—	—
*Cfr*I	30	30	30	2—25	—	—
*Cfr*91	1	1	1	30	—	—
*Eco*81I	21	21	21	5—25	—	—
*Pvu*II	44	44	44	2—30	—	—
*Sal*I	3	3	3	10—30	—	—
*Sma*I	1	1	1	30—40	—	—

* no additional fragment

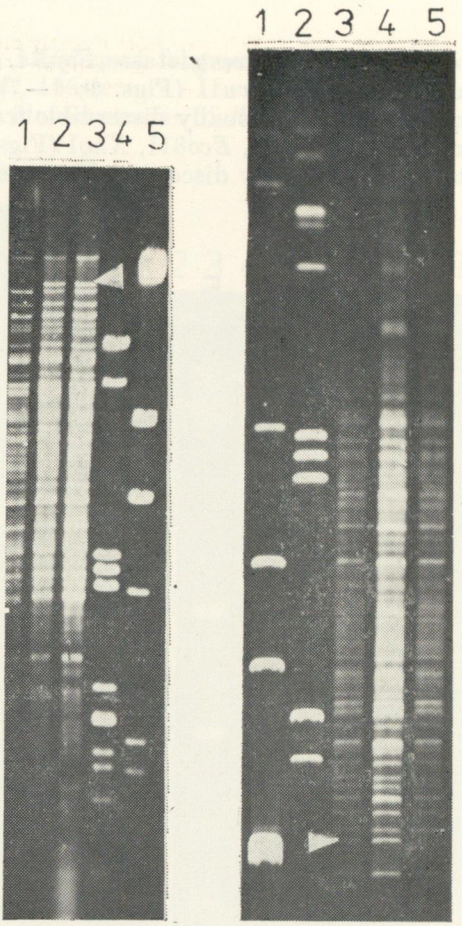

Fig. 1.
Restrictograms of *Rickettsia prowazekii* DNA cleaved by *Pst*I endonuclease

In the left — DNA of cloned strains, in the right — DNA of uncloned strains. Tracks 1, 2, and 3 — DNA of strains Breinl, Evir, E, respectively, cleaved by *Pst*I; track 4 — phage λ DNA cleaved by *Pst*I; track 5 — phage λ DNA cleaved by *Hind*III.

Here and thereafter the arrows indicate the additional DNA fragments. Cleavage and electrophoresis conditions are described in Materials and Methods.

The following restrictases were used: *Bcn*I, *Bsp*RI, *Cfr*I, *Cfr*91, *Cfr*13I, *Eco*RI, *Eco*81I, (*Sau*I), *Hind*III, *Msp*I, *Mva*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, *Xho*I, *Cfu*I (*Dpn*I), *Sau*3A ("Enzyme" Manufacturers, Vilnius, U.S.S.R.) as well as *BamH*I, *Bgl*I (Serva, F.R.G.).

Electrophoretic separation of DNA fragments was carried out in horizontal 0.6 % agarose gels (agarose type II, Sigma U.S.A.), first at 5 V/cm for 4 to 6 hr and then at 1 or 2 V/cm for 16—18 hr in tris-acetate buffer. Phage λ DNA (native and *Hind*III-cleaved) was used as a molecular standard.

The gels were stained with ethidium bromide (Sigma U.S.A.) They were photographed with Zenith E camera with orange light filter, film Micrat 300 and a UV radiation source (Desaga, F.R.G.).

Results

Each of the seventeen most commonly used restriction endonucleases yielded a characteristic and reproducible cleavage pattern (Figs. 1—10). The data of the analysis of restrictograms are presented in Table 2.

It can be seen that restrictases *Bsp*RI, *Cfr*I, *Cfr*13I, *Eco*RI, *Hind*III, *Msp*I, *Mva*I, *Pst*I, and *Pvu*II (Figs. 1; 4—7) cleaved the rickettsial DNA into a larger number of visually discernible fragments (from 30 to 51). Restrictases *Bam*HI, *Bcn*I, *Bgl*I, *Eco*81I, *Xho*I (Figs. 2, 3, 8) cleaved the rickettsial DNA into 8 to 21 visually discernible fragments. Cleavage with restrictases *Cfr*91,

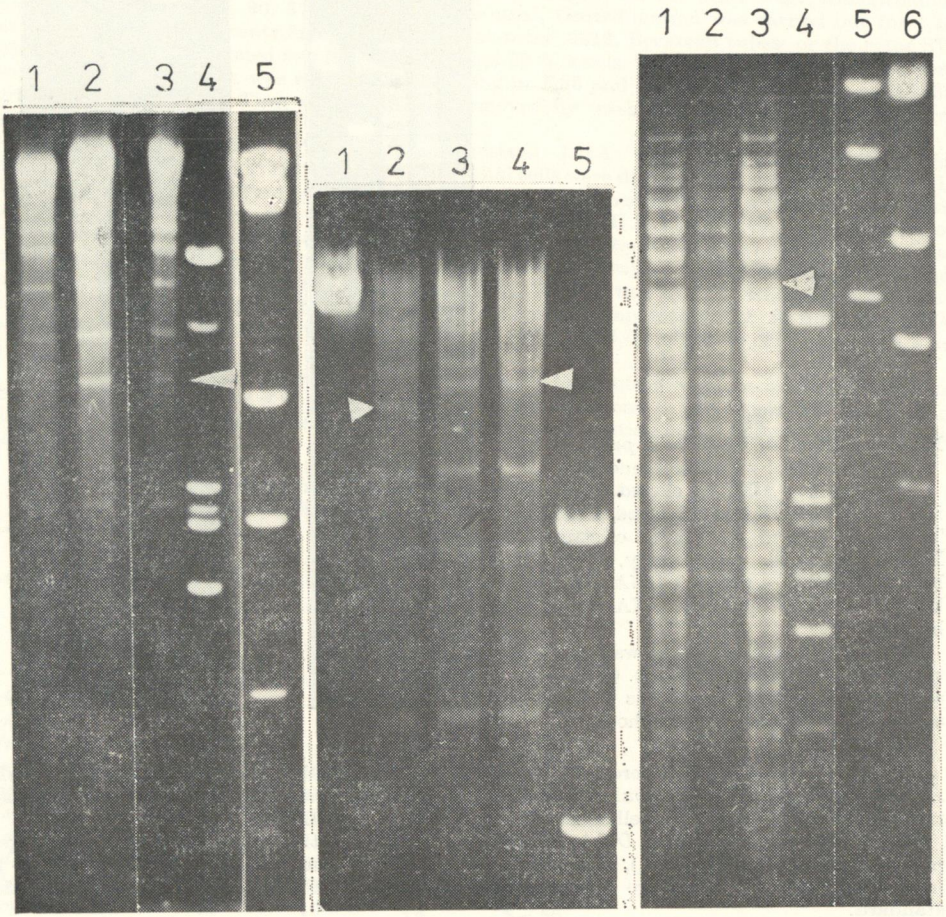

Fig. 2. **Fig. 3.** **Fig. 4.**

Fig. 2. Restrictograms of *Rickettsia prowazekii* cleaved by endonuclease *Bam*HI
Tracks 1, 2, 3, and 4 — the DNA of strains Breinl, Evir, E, and phage λ, respectively, cleaved by *Bam*HI; track 5 — phage λ DNA cleaved by *Hind*III.

Fig. 3. Restrictograms of *Rickettsia prowazekii* DNA cleaved by endonuclease *Bgl*I
Track 1 — phage λ DNA (native), tracks 2, 3, 4, 5 — the DNA of strains Breinl, Evir, E, phage λ, respectively, cleaved by BglI.

Fig. 4. Restrictograms of *Rickettsia prowazekii* cleaved by endonuclease *Cfr*I31
Tracks 1, 2, 3, 4 — DNA of strains Breinl, Evir, E, and phage λ, respectively, cleaved by *Cfr*I31; tracks 5 and 6 — phage λ DNA cleaved by *Eco*81I and *Hind*III, respectively.

*Sal*I, and *Sma*I yielded only a small number of fragments (Fig. 9). These data have been confirmed (Wood *et al.*, 1984).

Restrictograms of the DNA of both cloned strains of *Rickettsia prowazekii*

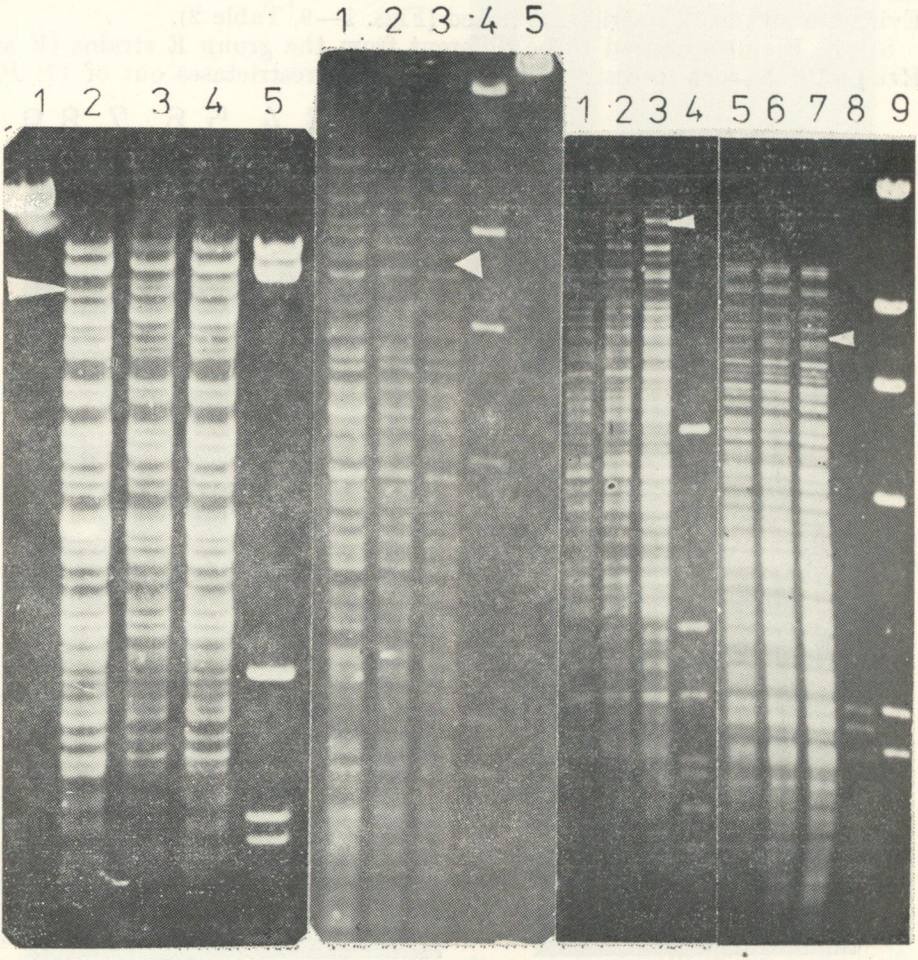

Fig. 5. **Fig. 6.** **Fig. 7.**

Fig. 5. Restrictograms of *Rickettsia prowazekii* DNA cleaved by endonuclease *Eco*RI
Track 1 — phage λ DNA (native); tracks 2, 3, 4, 5 — DNA of strains Breinl, Evir, E, phage λ, cleaved by *EcoR*I, respectively.

Fig. 6. Restrictograms of *Rickettsia prowazekii* DNA cleaved by endonuclease *Hind*III
Tracks 1, 2, 3, 4 — DNA of strains Breinl, Evir, E, phage λ, respectively, cleaved by *Hind*III; track 5 — phage λ DNA (native).

Fig. 7. Restrictograms of *Rickettsia prowazekii* cleaved by endonucleases *Mva*I and *Msp*I
Tracks 1, 2, 3, 4 — DNA of strains Breinl, Evir, E, phage λ, respectively, cleaved by *Mva*I; tracks 5, 6, 7, 8 — DNA of strains Breinl, Evir, E, phage λ cleaved by *Msp*I, respectively; track 9 — phage λ Dna cleaved by *Hind*III.

and those continuously passaged under standard conditions were totally identical with all the restrictases used (Fig. 1). It should be noted that the passage history of the cloned strain Breinl and of the uncloned one differed greatly, by more than 100 passages in CE (Table 1). The analysis of the restrictograms has shown identical DNA restriction patterns for strains E and Evir with all the 17 restrictases tested (Figs. 1—9, Table 2).

Strain Breinl appeared to be different from the group E strains (E and Evir) with respect to restriction patterns by 9 restrictases out of 17: *Pst*I

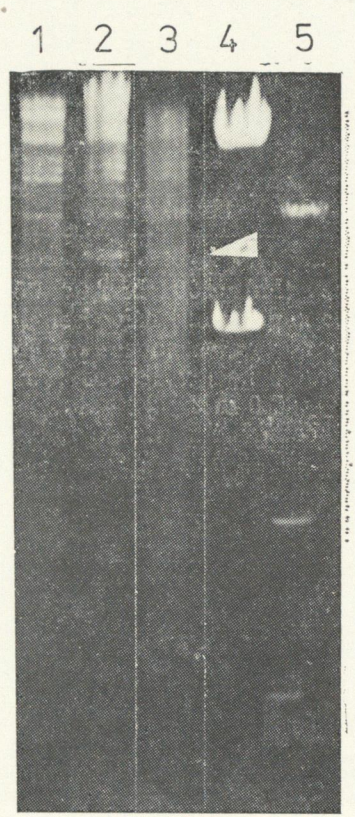

Fig. 8.

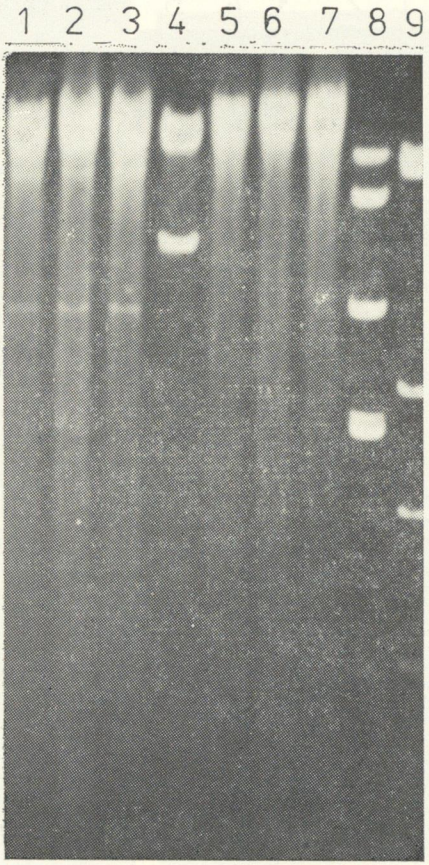

Fig. 9.

Fig. 8. Restrictograms of *Rickettsia prowazekii* cleaved by endonuclease XhoI
Tracks 1, 2, 3, 4 — DNA of strains Breinl, Evir, E, phage λ, respectively, cleaved by *Xho*I; track 5 — phage λ DNA cleaved by *Hind*III.

Fig. 9. Restrictograms of *Rickettsia prowazekii* DNA cleaved by endonucleases *Sal*I and *Sma*I
Tracks 1, 2, 3, 4 — DNA of strains Breinl, Evir, E, phage λ, respectively, cleaved by *Sal*I; tracks 5, 6, 7, 8 — DNA of strains Breinl, Evir, E, phage λ, respectively, cleaved by *Sma*I; track 9 — phage λ DNA cleaved by *Hind*III.

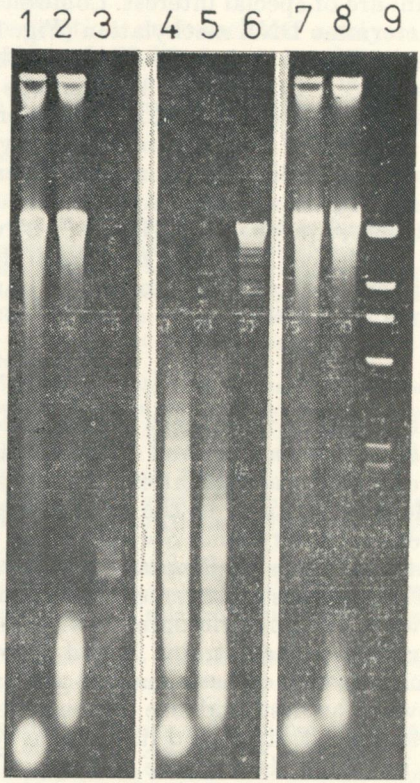

Fig. 10.
Restrictograms of *Rickettsia prowazekii* cleaved by *Sau*3A and *Cfu*I
Tracks 1, 2 — DNA of strains Evir and Breinl (native); tracks 3, 4, 5 — DNA of phage λ, strains Evir, Breinl, respectively, cleaved by *Sau*3A; tracks 6, 7, 8 — DNA of phage λ, strains Evir, Breinl, respectively, cleaved by *Cfu*I; track 9 — phage λ DNA cleaved by *Hind*III.

(Fig. 1), *Bam*HI (Fig. 2), *Bgl*I (Fig. 3), *Cfr*13I (Fig. 4), *Eco*RI (Fig. 5), *Hind*III (Fig. 6), *Msp*I, *Mva*I (Fig. 7), *Xho*I (Fig. 8). In all cases these differences concerned 1 or 2 bands in the restrictogram and were in the range of 8 to 20 thousand nucleotide pairs (tnp, Table 2). Thus, for example, strain Breinl was found after *Bgl*I cleavage to have an additional band in the 20 tnp range found neither at E nor at Evir (Fig. 3); this band was in 9 tnp range by *Hind*III cleavage (Fig. 6). By *Cfr*13I cleavage it was in the 8 tnp range (Fig. 4), by *Msp*I — in the 9 tnp range (Fig. 7), by *Mva*I — in the 18 tnp range (Fig. 7), and with *Pst*I — in the 15 tnp range (Fig. 1). Group E strains (E and Evir) also had an additional band (about 9.5 tnp) not found in strain Breinl digest after the cleavage with *Bam*HI (Fig. 2), by *Bgl*I the additional band was about 23 tnp (Fig. 3), by *Eco*RI about 18 tnp (Fig. 5), by *Msp*I — 8 tnp (Fig. 7), by *Pst*I about 20 and 16 tnp, respectively (Fig. 1), and by *Xho*I — 15 tnp (Fig. 8). The data obtained with *Bam*HI were also confirmed by others: during cleavage of the DNA strain Breinl and strain E DNA with *Bam*HI an additional band of about 9.55 tnp was found in the strain E digests (Wood *et al.*, 1984; Regnery *et al.*, 1985).

The *Cfu*I and *Sau*3A cleavage of rickettsial DNA from strains Evir and Breinl are of special interest. Combined application of these restrictases helps to determine DNA methylation (Fig. 10) as they recognize the same sequence 5′... GATC ... 3′. but *Cfu*I cleaves this sequence only if it is methylated in adenine 5′ ... GATC ... 3′, (Maniatis *et al.*, 1982; Kessler *et al.*, 1985). The action of restrictase *Sau*3A is independent of methylation. The character of the cleavage of the DNA of strains Evir and Breinl with *Sau*3A indicated the presence of a large amount of sequences 5′... GATC ...3′ in the rickettsial DNA (Fig. 10, tracks 4 and 5). The absence of cleavage with *Cfu*I showed that the sequence 5′ ... GATC ... 3′ was not methylated (Fig. 10, tracks 7 and 8). Hence, *Rickettsia prowazekii* do not possess a methylase activity with a site-specificity similar to that of dam-methylase of *Escherichia coli* (Hattman *et al.*, 1978).

Discussion

It should be noted that the restrictograms of the DNA of cloned strains of *Rickettsia prowazekii* and those continuously passaged under standard conditions are totally identical. Thus, such rickettsial DNA probably does not undergo variations in the course of passages. This result was confirmed by others (Regnery and Spruill, 1984) and is of methodological importance for the analysis of *Rickettsia prowazekii* DNA cleavage.

The genomes of isogenic strains E and Evir failed to reveal any differences between their restrictograms with any of the 17 restrictases tested. It has been shown that strains E and Evir, though showing stable differences in virulence, do not have marked genetic structural differences. These findings provide the first evidence for isogenicity of the weakly pathogenic *Rickettsia prowazekii* strain E and its virulent revertant strain Evir. They also confirm the biological data on the isolation of strain Evir (Balayeva, 1971). Cleavage of the virulent strain Madrid I DNA, the parent strain of strain E (Clavero and Perez-Gallardo, 1944; Perez-Gallardo, 1963) would be of value for the study of the variability of the weakly pathogenic *Rickettsia prowazekii* strain E.

The differences between restrictograms of strain Breinl and group E strains (E and Evir) have been observed during cleavage with 9 endonucleases. Up to the present, evidence for the differences between strain E and strain Breinl has been obtained with restrictases *Sac*I and *Bam*HI (Regnery *et al.*, 1983, 1985; Wood *et al.*, 1984). We have detected an additional fragment in the strain E cleavage pattern with *Bam*HI and the same additional fragment (about 9 tnp) in the strain Evir isogenic to strain E. Complete identity of restrictograms of isogenic strains E and Evir which, however, differed in virulence, and their differences from reference strain Breinl seemed to reflect the strain peculiarities unrelated to virulence.

It should be noted that differences in DNA restriction between strain Breinl and group E strains (E and Evir) only concern 1 or 2 fragments, whereas in general the restriction patterns of all three strains show a marked similarity. This confirms the genetic conservation of the genome of *Rickettsia*

prowazekii described in previous papers (Regnery and Spruill, 1984; Wood *et al.*, 1984; Regnery *et al.*, 1983, 1985, 1986).

The data obtained indicate that restriction endonuclease analysis has good prospects for further differentiation of *Rickettsia prowazekii* strains.

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References

Balaeva, N. M. (1969): Rise in virulence of vaccinal strain E of *Rickettsia prowazekii* in the course of passages in albino mice lungs. *Vestn. Akad. med. Nauk* **1969** (**10**), 51–56 (in Russian).

Balaeva, N. M. (1971): On the virulence of vaccinal strain E of *Rickettsia prowazekii* and some biological properties of typhus. Doctor's Thesis, The N. F. Gamaleya Research Institute of Epidemiology and Microbiology, U.S.S.R., Academy of Med. Sci., Moscow (in Russian).

Balaeva, N. M., and Nikolskaya, V. N. (1973*a*): Rice in virulence of vaccinal strain E of *Rickettsia prowazekii* in the course of passages in albino mice lungs and in guinea pigs. *Zh. Gigien. Epidemiol. Mikrobiol. Immunol.* **17**, 11–19 (in Russian).

Balaeva, N. M., and Nikolskaya, V. N. (1973*b*): Analysis of the lung culture of *Rickettsia prowazekii* strain E based on the ability to increase virulence in the course of passages in albino mice lungs. *Zh. Gigien. Epidemiol. Mikrobiol. Immunol.* **17**, 274–283 (in Russian).

Balaeva, N. M., Gudima, O. S., and Nikolskaya, V. N. (1976): The changeability of strain E of *Rickettsia prowazekii* in tissue culture – chick embryos. *Folia Microbiol.* **21**, 500.

Clavero, C. G., and Perez-Gallardo, F. P. (1944): Experimental study of a non-pathogenic immunizing strain of *Rickettsia prowazekii*. *Trop. Dis. Bull.* **41**, 24 (Revista de Sanidad e Hygiene Publica, 1943, 17).

Gambrill, M. R., and Wisseman, C. L. (1973): Mechanism of immunity in typhus infection. II. Multiplication of typhus rickettsiae in human macrophage cell culture in the nonimmune system: influence of virulence of rickettsial strains and of chloramphenicol. *Infect. Immun.* **8**, 519–527.

Hattman, S., Brooks, J. E., and Masurekar, M. (1978): Sequence specificity of the PI modification methylase (M *Eco*PI) and the DNA methylase (M *Ecodam*) controlled by the *Escherichia coli* dam gene. *J. mol. Biol.* **126**, 367–380.

Ignatovich, V. F. (1975): Enhancement of the antigenic activity and virulence of the vaccine strain E of *Rickettsia prowazekii* by passages in cell culture. *Acta virol.* **19**, 481–485.

Kazár, J., Brezina, R., and Úrvölgyi, J. (1973): Studies on the E strain of *Rickettsia prowazekii*. *Bull. Wrld. Health Org.* **49**, 257–265.

Kessler, C., Neumaier, T. S., and Wolf, W. (1985): Recognition sequences of restriction endonucleases and methylases – a review. *Gene* **33**, 1–102.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982): *Molecular cloning.* A Laboratory Manual. Cold Spring Harbor Laboratory, U.S.A.

Oaks, E. V., Wisseman, C. L., and Smith, J. F. (1981): Radiolabelled polypeptides of *Rickettsia prowazekii* grown in microcarrier cell cultures, pp. 461–472. In W. Burgdorfer, R. L. Anacker (Eds): *Rickettsiae and Rickettsial Diseases*, Academic Press, Inc., New York.

Perez-Gallardo, F. P. (1963): Study of the E strain isolated in Madrid in 1941. Its antigenic properties, its avirulence. *Bull. Soc. Path. Exot.* **56**, 805–822.

Perez-Gallardo, F. O., and Fox, Y. P. (1948): Infection and immunization of laboratory animals with *Rickettsia prowazekii* of reduced pathogenicity, strain E. *Am. J. Hyg.* **48**, 6–21.

Priefer, U., Simon, R., and Pühler, A. (1984): *Advanced Molecular Genetics*, 197 p.

Pshenichnov, V. A., Vorobiev, A. A., and Balaeva, N. M. (1976): *Attenuation of Viruses and Rickettsiae*, Meditsina, Moscow.

Regnery, R. L., Tzianabos, T., Esposito, J. J., and McDade, J. E. (1983): Strain differentiation of epidemic typhus rickettsiae (*R. prowazekii*) by DNA restriction endonuclease analysis. *Current Microbiol.* **8**, 355–358.

Regnery, R. L., and Spruill, C. L. (1984): Extent of genetic heterogeneity among human isolates of *Rickettsia prowazekii* as determined by restriction endonuclease analysis of rickettsial DNA, pp. 297–300. In L. Lewe, D. Schlissenger (Eds): *Microbiology* **84**, American Society of Microbiology.

Regnery, R. L., Spruill, C. L., and Wood, D. O. (1985): Analysis of rickettsial DNA by hybridization with cloned rickettsial DNA probes, pp. 62—71. In J. Kazár (Ed.): *Rickettsiae and Rickettsial Diseases.* Proc. IIIrd International Symposium on Rickettsiae and Rickettsial Diseases, Bratislava.

Regnery, R. L., Fu, Z. Y., and Spruill, C. L. (1986): Flying squirrel-associated *Rickettsia prowazekii* (Epidemic Typhus Rickettsiae) characterized by a specific DNA fragment produced by restriction endonuclease digestion. *J. clin. Microbiol.* **23**, 189—191.

Silverman, D. Y., and Wisseman, C. L. (1978): Ultrastructure studies on typhus and spotted fever rickettsiae with special emphasis on the extracellular layers, pp. 37—48. In J. Kazár (Ed.): *Rickettsiae and Rickettsial Diseases*, Proc. IIIrd International Symposium on Rickettsiae and Rickettsial Diseases, Bratislava.

Turco, J., and Winkler, H. H. (1982): Differentiation between avirulent strains of *Rickettsia prowazekii* by macrophage-like cell lines. *Infect. Immun.* **35**, 783—791.

Weiss, E., Rees, H. B., Hayes, Jr., and Hayes, J. R. (1967): Metabolic activity of purified suspension of *Rickettsia rickettsii*. *Nature* **213**, 1020—1022.

Weiss, E., Coolbaugh, J. C., and Williams, J. C. (1975): Separation of viable *Rickettsia typhi* from yolk sac and L-cell host components by renografin density gradient centrifugation. *Appl. Microbiol.* **30**, 456—463.

Winkler, H. H., and Miller, E. T. (1980): Phospholipase A activity in the hemolysis of sheep and human erythrocytes by *Rickettsia prowazekii*. *Infect. Immun.* **29**, 316—321.

Winkler, H. H., and Daugherty, R. M. (1983): Cytoplasmic distinction of avirulent and virulent *Rickettsia prowazekii* fusion of infected fibroblasts with macrophage-like cells. *Infect. Immun.* **40**, 1245—1247.

Wolbach, S. B., Todd, J. Z., and Palfrey, F. W. (1922): *The Etiology and Pathology of Typhus*, Harvard University Press, Cambridge, Mass., U.S.A.

Wood, D. O., Sikorski, R. S., Atkinson, W. H., Krause, D. C., and Winkler, H. H. (1984): Cloning *Rickettsia prowazekii* genes in *Escherichia coli* K-12, pp. 301—304. In L. Lewe, D. Schlissenger (Eds): *Microbiology* **84**, American Society of Microbiology.