Reverting and Non-Reverting Rough Variants of *Bacillus anthracis*

By ELINOR W. MEYNELL

*Wright-Fleming Institute of Microbiology, St Mary's Hospital, Paddington,
London, W. 2*

(Received 20 November 1962)

SUMMARY

Non-capsulated variants of *Bacillus anthracis* have been said not to revert to the capsulated state. However, in the present work, capsulated revertants were isolated from about half the non-capsulated strains tested either by exposure to phage W α , which attacks only non-capsulated organisms, or by passage in mice.

INTRODUCTION

Virulent strains of *Bacillus anthracis* form mucoid colonies of capsulated organisms when grown under appropriate conditions (Nungester, 1929; Schaefer, 1936; Sterne, 1937 *a, b*; Thorne, Gomez & Housewright, 1952). After several days' incubation, these colonies may show rough outgrowths which contain non-capsulated avirulent variants presumably arisen by mutation (Stamatin, 1934; Schaefer, 1936; Sterne, 1937 *a, b*; Chu, 1952). Examination of rough variants isolated from many different strains of *B. anthracis* has led to the belief that they never revert to the virulent capsulated type, even on animal passage (Bail, 1915; Sterne, 1937 *a, b*; Chu, 1952); and since they retain the toxigenic (see Smith & Keppie, 1955), and thus the immunizing, activity of the parental type (Bail, 1915; Stamatin & Stamatin, 1936; Stamatin, 1937), they have been widely and successfully used during the last 25 years for the immunization of domestic animals (Sterne, 1939 *a*).

Non-reverting mutants are especially convenient for genetic experiment, since parental types can only appear following recombination. Consequently, various known methods of gene transfer were tested for their ability to transmit the C+ genes which determine capsule production to a rough (C–) recipient strain. Any C+ recombinants were to be selected by phage W α , which attacks non-capsulated, but not capsulated, organisms (McCloy, 1951). This experiment led to the unexpected finding of mucoid colonies on control plates which had received only bacteria of the non-capsulated (C–) strain, and phage W α . These colonies could not have arisen by transduction (Zinder & Lederberg, 1952), since the phage stock had been grown on a genetically identical strain. Thus it seemed that, contrary to what has been generally accepted, at least some C– variants were able to revert to the C+ form. Further tests on a number of rough variants arising independently from several anthrax strains have shown that many, though not all, revert to the capsulated virulent form. It should be added that each of the C– variants was purified by three successive single spore isolations before being tested for reversion, so as to

exclude contamination by C+ organisms of the parental culture. Thorne (1960) briefly mentioned the appearance of C+ colonies in rough cultures exposed to phage, and Wright, Puziss & Neely (1962) observed the emergence of C+ clones while studying the formation of protective antigen by C- strains in a chemically defined medium.

METHODS

Media. Nutrient broth was made of Lab. Lemco 0.1% (w/v), Tryptone (Oxoid) 0.2% (w/v), Peptone (Oxoid) 1% (w/v) and NaCl 0.7% (w/v). Nutrient agar contained nutrient broth solidified with 1.5% (w/v) Davis N.Z. agar. Cultures incubated in air, particularly those used for the production of spore suspensions, were often grown on plates containing 2 parts nutrient agar and 1 part 1.5% (w/v) solution of liver extract (Oxoid). For production of capsules, the bacteria were grown on nutrient agar containing either 20% (v/v) horse serum or 0.025 M-NaHCO₃ and incubated in air + CO₂ (i.e. about 30% of the air replaced by CO₂).

Spore suspensions. A confluent growth of bacteria on liver agar was incubated for several days at 37° and then suspended in distilled water and incubated for some further days (Hardwick & Foster, 1952). Suspensions were sometimes lightly centrifuged to remove clumps; only those in which the spores were well dispersed and in which no vegetative bacteria could be seen were finally used.

Phage stocks. Stocks of phage Wα grown on non-capsulated variants of the atypical strain Davis (McCloy, 1958) were used in most experiments, but occasionally phage grown on two other strains was used, as described in the next section. Lysates were freed from bacteria by filtration through Seitz filters, and had titres of 5×10^9 to 2×10^{10} plaque-forming particles/ml.

Strains of Bacillus anthracis tested. Isolation and purification of C- clones. Eleven strains tested were received in the C- state and produced only rough non-mucoid colonies on serum or bicarbonate agar incubated in air + CO₂. These comprised: (1) 2 strains used as vaccines, CN 1874 and CN 3518 (respectively 34F2 and v8R1 from Ondestepoort, South Africa), received from Mr H. Proom, The Wellcome Research Laboratories, Beckenham, Kent; (2) 7 strains received from Dr G. Ivánovics, Universitatis Medicae Szegediensis, Szeged, Hungary, 5 of which had been reported as non-reverting to the capsulated state (Ivánovics & Földes, 1958); (3) 1 strain received from Dr J. Davis, Public Health Laboratory, County Hall, Westminster Bridge, London; (4) 1 strain from the author's stocks. Nine strains when received produced a mixture of mucoid and rough colonies, or only mucoid colonies; C- variants were then isolated by picking a rough colony or a rough outgrowth from a mucoid colony. The C- strains were rigorously purified in view of Sterne's (1937*a, b*) opinion that any apparent reversions to capsulation observed either by himself or other workers (Schaefer, 1936) were in reality the result of incomplete purification of the rough isolate, which had not been entirely freed from capsulated bacteria carried over from the parent strain. Three successive single-colony isolations were therefore made by allowing the C- clone to sporulate, plating the resultant spore suspension for single colonies, and repeating the procedure twice more before accepting a C- clone as pure. This ensured that, in each cycle, each colony arose from a single spore and not from entangled chains of vegetative forms.

Isolation of C+ bacteria from C- strains. Serum or bicarbonate agar plates were spread with 0.2 ml. of undiluted phage W α preparation and 10^8 – 10^9 spores of the C- strain, and then, as quickly as possible, transferred to an anaerobic jar in which 20–30 % (v/v) of the air was then replaced with CO $_2$. Spores, which do not adsorb the phage, were used as inocula, since it was thought that in this way any C+ organisms which might be present would remain resistant to the phage until they reached the atmosphere of CO $_2$ in which they could develop the protective capsule. Mucoid colonies which appeared were subcultured several times to free them from contaminating phage, and before being tested for virulence were shown to be phage-sensitive (on ordinary agar in air) and non-lysogenic (Ivánovics, 1962).

Virulence tests. The C+ strains were tested at doses of about 10^4 , 10^3 , 10^2 and 10 spores, and the C- at doses of about 10^7 , 10^6 , 10^5 and 10^4 spores, three mice being used per dose which was given intraperitoneally in 0.2 ml. sterile distilled water. At the same time, viable counts were made on ordinary agar and on serum or bicarbonate agar incubated in air + CO $_2$; the C+ strains produced an occasional C- colony, but with the C- strains, only rough non-mucoid colonies were seen. Mice which died were examined *post mortem*, and a sample of heart blood cultured on serum or bicarbonate agar in air + CO $_2$.

RESULTS

Selection with phage grown on the same strain. Non-capsulated (C-) variants were isolated from the C+ strains 2160s and Davis (McCloy, 1958). These strains are asporogenous and greatly attenuated from causes other than the absence of a capsule. Strain 2160s is a smooth colony variant (Nungester, 1929; Sterne, 1938) isolated from a virulent strain, 2160, by growth in broth containing calcium chloride (Renaux, 1952). Since these strains did not form spores, the C- variants were simply put through three successive single-colony isolations with plating on serum agar in air + CO $_2$ in order to detect any possible mucoid sectors in the colonies. The third strain tested, 1444, produced spores. Stocks of phage were grown on each of these three C- strains, and each strain yielded a small proportion (about 10^{-7}) of C+ colonies when plated with phage which had been grown on itself. Since the strain tested and the last host of the phage were identical, the C+ colonies could only have arisen by selection, and this conclusion was confirmed for strain 1444 by isolating C+ bacteria from the blood of mice fatally infected with large doses of the C- strain. Since strains 2160s and Davis were virtually avirulent even in the C+ state, no attempts were made to isolate C+ from C- bacteria of these strains by animal passage.

Tests with phage grown on C- strain Davis. Table 1 shows the results of tests on 30 C- variants isolated from 21 strains of *Bacillus anthracis*. C+ bacteria were found in 17 of the 30 C- cultures at frequencies of 7×10^{-9} to 10^{-6} . Eleven of the C- variants were also inoculated into mice, and all but one produced some fatal infections in high doses of 10^6 or more spores. With 5 of the 11 strains, C+ bacteria were found in the heart blood *post mortem*; fatal infections with the other 5 yielded only C- bacteria.

Differences between independent C- isolates. The most obvious difference was that C+ revertants were obtained from some, but not all, C- variants, even when these were isolated from the same C+ parental strain. When several independent C-

variants were isolated from five C+ strains, the fractions giving C+ revertants were $\frac{1}{3}$, $\frac{1}{3}$, $\frac{2}{3}$, $\frac{2}{3}$, and $\frac{1}{2}$, respectively. The change C+ to C- thus seems to resemble variation in other characters which may or may not revert, where the non-reverting mutation is often due to deletion or inversion in the genome (see Demerec & Hartman, 1959).

Table 1. *Tests with phage Wα grown on C- Bacillus anthracis strain Davis*

Strains received as	No. of strains	No. of independent C- isolates tested	No. of C- variants yielding C+		
			(i) with phage	(ii) in mice	(iii) total
C+	10	19	12/19*	3/4	12/19
C-	11	11	5/11	2/7	5/11

* Number of strains tested.

C- variants appear more readily in some C+ strains than in others (Sterne, 1937*b*) which is probably at least in part because C- bacteria can grow out from the mucoid parent colony more readily when they form long chains. Typical rough outgrowths were never seen with one of the C+ strains examined, but only indentations or narrow borders of non-mucoid growth around the mucoid colonies. The reason for this was most probably that, under conditions unsuitable for capsule production, the colonies of this strain were rather smooth instead of having the rough form associated with long chains of bacteria. These non-mucoid areas contained organisms which killed about 50% of mice given 10^5 spores, although there was no evidence from microscopic examination that traces of capsule were still present, as found by Schaefer (1936) in some of his variants. Highly mucoid C+ revertants were found in some fatally infected mice, and could also be isolated by culture with phage Wα.

DISCUSSION

Conditions favouring the production of mucoid colonies and short bacillary chains by capsulated (C+) forms of *Bacillus anthracis* are considered to confer a selective advantage on non-capsulated (C-) variants which eventually project outwards from the colony towards new sources of nutrient (Chu, 1952). C+ revertants are not seen when C- bacteria are simply cultured in air+CO₂, which may partly explain why C- variants have so long been stated to be non-reverting. Another observation which supported this belief was the absence of fatal infections in cattle and other animals vaccinated with C- cultures (Sterne, 1939*a, b*). The present results show that at least half the C- variants isolated from C+ strains revert to C+, and also that the kind of C- variant produced (reverting or non-reverting) is not determined by the C+ parental strain. C+ revertants are recovered only when C- cultures are exposed to a selective agent like phage Wα, which attacks non-capsulated, but not capsulated, bacteria (McCloy, 1951), or to the anti-bacterial mechanisms present in mice which preferentially kill C- organisms. Mice may select C+ bacteria more efficiently than other animals, for their immunity to anthrax may depend on anticapsular antibody (Tomcsik & Ivánovics, 1938; Ivánovics, 1938; Sterne 1939*b*; Gladstone, 1946), unlike other animals such as the guinea-pig, rabbit or sheep (Stamatin, 1937; Tomcsik & Ivánovics, 1938;

Ivánovics, 1938; Sterne, 1939*a, b*; Gladstone, 1946; Staub & Grabar, 1943; Smith, Zwartouw & Harris-Smith, 1956). Moreover, in the guinea-pig at least, immunity develops so rapidly after a dose of C — spores (Sterne, 1939*b*) that C + revertants might not be able to cause a fatal infection if they did not arise soon after inoculation.

Schaefer (1936), reported the isolation of unstable, as well as of stable, non-capsulated variants, but no revertants were recovered from several rough variants examined by Stamatin (1934, 1937; Stamatin & Stamatin, 1936), from more than nine variants isolated by Sterne (1937*b*, 1939*a*) and from the many more tested by Chu (1952). This is surprising in view of the present results, but it must be mentioned that the two vaccine strains, CN 1874 and CN 3518, originally from Dr Sterne's collection, did not revert in the present tests. These and other rough variants used for active immunization are not likely to have been derived from C + strains that gave only non-reverting C — variants. Dr Sterne tells me that his C + strains were chosen at random, and that the explanation may be that he tested as possible vaccines only those C — cultures which remained free from capsulated bacteria, and dismissed any C + bacteria as contaminants carried over from the original C + parental strain.

REFERENCES

- BAIL, O. (1915). Veränderungen der Bakterien im Tierkörper. IX. Über die Korrelation zwischen Kapselbildung, Sporenbildung und Infektiosität des Milzbrandbacillus. *Zbl. Bakt. (I. Abt. Orig.)*, **75**, 159.
- CHU, H. P. (1952). Variation of *Bacillus anthracis* with special reference to the non-capsulated avirulent variant. *J. Hyg., Camb.* **50**, 433.
- DEMEREK, M. & HARTMAN, P. E. (1959). Complex loci in microorganisms. *Annu. Rev. Microbiol.* **13**, 377.
- GLADSTONE, G. P. (1946). Immunity to anthrax: protective antigen present in cell-free culture filtrates. *Brit. J. exp. Path.* **27**, 394.
- HARDWICK, W. A. & FOSTER, J. W. (1952). On the nature of sporogenesis in some aerobic bacteria. *J. gen. Physiol.* **35**, 907.
- IVÁNOVICS, G. (1938). Über die Milzbrandimmunität. *Z. Immun-Forsch.* **94**, 436.
- IVÁNOVICS, G. (1962). The pathogenicity of *Bacillus anthracis* lysogenic with mutants of phage W. *J. gen. Microbiol.* **28**, 87.
- IVÁNOVICS, G. & FÖLDES, J. (1958). Problems concerning the phylogenesis of *Bacillus anthracis*. *Acta microbiol. hung.* **5**, 89.
- MCCLOY, E. W. (1951). Studies on a lysogenic *Bacillus* strain. I. A bacteriophage specific for *Bacillus anthracis*. *J. Hyg., Camb.* **49**, 114.
- MCCLOY, E. W. (1958). Lysogenicity and immunity to *Bacillus* phage W. *J. gen. Microbiol.* **18**, 198.
- NUNGESTER, W. J. (1929). Dissociation of *B. anthracis*. *J. infect. Dis.* **44**, 73.
- RENAUX, E. (1952). Culture de *Bacillus anthracis* en milieu calcique et en milieu oxalaté. *Ann. Inst. Pasteur*, **83**, 38.
- SCHAEFER, W. (1936). Dissociation de la bactérie charbonneuse. *C.R. Soc. Biol., Paris*, **122**, 1178.
- SMITH, H. & KEPPIE, J. (1955). Studies on the chemical basis of the pathogenicity of *Bacillus anthracis* using organisms grown *in vivo*. *Symp. Soc. gen. Microbiol.* **5**, 126.
- SMITH, H., ZWARTOUW, H. T. & HARRIS-SMITH, P. W. (1956). The chemical basis of the virulence of *Bacillus anthracis*. VIII. Fractionation of the intracellular material of *Bacillus anthracis*. *Brit. J. exp. Path.* **37**, 361.
- STAMATIN, N. (1934). Contributions a l'étude de la morphologie et la biologie de la bactérie charbonneuse. *Arh. Vet., Buch.* **26**, 1.
- STAMATIN, N. (1937). L'immunisation anticharbonneuse au moyen d'une souche de *Bacillus anthracis* acapsulogène chez le mouton. *C.R. Soc. Biol., Paris*, **125**, 90.

- STAMATIN, N. & STAMATIN, L. (1936). Le pouvoir immunisant de souches acapsulogènes de *Bacillus anthracis*. *C.R. Soc. Biol., Paris*, **122**, 491.
- STAUB, A-M. & GRABAR, P. (1943). Recherches immunochimiques sur la bactériodie charbonneuse. Rôle de la capsule dans l'immunisation anticharbonneuse. *C.R. Soc. Biol., Paris*, **137**, 623.
- STERNE, M. (1937*a*). Variation in *Bacillus anthracis*. I. Some effects of carbon dioxide on the formation of capsules and spores by *B. anthracis*. II. Some correlations between colony variation and pathogenicity in strains of *B. anthracis*. *Onderstepoort J. vet. Sci.* **8**, 271.
- STERNE, M. (1937*b*). The effects of different carbon dioxide concentrations on the growth of virulent anthrax strains. Pathogenicity and immunity tests on guinea-pigs and sheep with anthrax variants derived from virulent strains. *Onderstepoort J. vet. Sci.* **9**, 49.
- STERNE, M. (1938). Variation in the colony form of the anthrax bacillus. *Onderstepoort J. vet. Sci.* **10**, 245.
- STERNE, M. (1939*a*). The use of anthrax vaccines prepared from avirulent (uncapsulated) variants of *Bacillus anthracis*. *Onderstepoort J. vet. Sci.* **13**, 307.
- STERNE, M. (1939*b*). The immunisation of laboratory animals against anthrax. *Onderstepoort J. vet. Sci.* **13**, 313.
- THORNE, C. B. (1960). Biochemical properties of virulent and avirulent strains of *Bacillus anthracis*. *Ann. N.Y. Acad. Sci.* **88**, 1024.
- THORNE, C. B., GOMEZ, C. G. & HOUSEWRIGHT, R. D. (1952). Synthesis of glutamic acid and glutamyl polypeptide by *Bacillus anthracis*. II. The effect of carbon dioxide on peptide production on solid medium. *J. Bact.* **63**, 363.
- TOMCSIK, J. & IVÁNOVICS, G. (1938). Die Schutzwirkung des Milzbrand-Antikapsel-Immunkörpers gegenüber der Milzbrand-infection. *Z. Immun.-Forsch.* **94**, 28.
- WRIGHT, G. G., PUZISS, M. & NEELY, W. B. (1962). Studies on immunity in anthrax. IX. Effect of variations in cultural conditions on elaboration of protective antigen by strains of *Bacillus anthracis*. *J. Bact.* **83**, 515.
- ZINDER, N. D. & LEDERBERG, J. (1952). Genetic exchange in *Salmonella*. *J. Bact.* **64**, 679.