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CALCIUM REQUIREMENT AND VIRULENCE OF *YERSINIA ENTEROCOLITICA*

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SUMMARY. At an optimal concentration of magnesium, highly virulent wild strains of *Yersinia enterocolitica* serotype O:8, with an LD₅₀, for mice, of $<10^3$ cells intravenously, had an in-vitro requirement for calcium at 37°C but not at 26°C. Avirulent wild strains of *Y. enterocolitica* (LD₅₀ $>10^7$ cells intravenously) did not have this calcium dependence. When grown on calcium-depleted media at 37°C, eight highly virulent strains yielded 0.5–6% large calcium non-requiring, avirulent colonies; the remaining colonies were slow growing, calcium dependent and highly virulent. Like wild avirulent strains, these calcium non-requiring mutants were quickly destroyed in organs within 48 h, even after large intravenous challenge. In contrast the slow-growing calcium-dependent colonies were highly virulent on intravenous inoculation, growing rapidly in the liver, spleen and lungs to produce multiple abscesses. Homogenates of heavily infected organs produced the original proportion of calcium non-requiring colonies when plated on media without calcium. Results of a fluctuation test suggested that the emergence of calcium non-requiring mutants is the result of induction rather than spontaneous mutation.

INTRODUCTION

Yersinia enterocolitica infection in man can produce acute suppurative gastroenteritis (Niléhn, 1969; Mollaret, 1971; Knapp *et al.*, 1973) or systemic disease such as arthritis, osteitis, erythema nodosum and septicaemia (Mollaret, 1971; Winblad, 1973). Most isolates from blood or stools of patients have a very low virulence in mice (Mollaret and Guillon, 1965) but some strains, usually of serotype O:8 are highly virulent in mice (Carter, Varga and Keet, 1973; Quan *et al.*, 1974). These strains

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multiply rapidly in the organs of infected animals, and invade all the tissues, producing abscesses (Carter and Collins, 1974). The LD₅₀ of these strains after intravenous infection is often less than 10³ bacteria per mouse (Carter, 1975).

In the genus *Yersinia*, highly virulent strains usually belong to the species *Y. pestis*. When such strains are subcultured on medium without calcium, they may show a high mutation rate to avirulence of 0.1–1% (Higuchi, Kupferberg and Smith, 1959; Higuchi and Smith, 1961; Brubaker and Surgalla, 1964; Brubaker, 1972). The same phenomenon has recently been demonstrated for three highly virulent strains of *Y. enterocolitica* (Carter, Zahorchak and Brubaker, 1980; Gernski, Lazere and Casey, 1980).

In this study we present a comparison of the effect of calcium depletion on highly virulent and avirulent wild strains of *Y. enterocolitica*.

MATERIALS AND METHODS

Bacteria. Each of the 26 wild strains of *Y. enterocolitica* used in this work was isolated from a human source. All of them, except three of undetermined serotype, were of serotype O:8. The strains used were: strain WA (ATCC27729, NCTC10938, Institut Pasteur YE2705) previously described (Carter *et al.*, 1973); strain BR and strain SH, from Dr G. Preiser (Delaware Valley Hospital, Walton, NY); strains TFW6331 and TFW6324, from Dr T. F. Wetzler (State of Washington Laboratory); strains E44, E217, E2380, E2383, E2397, E2574, E2739, E2746, E2978, E3192, E3193 from Dr M. Shayegani (New York State Laboratory); strains 80-748001-01 (undetermined serotype), 80-748001-09, 80-748001-19, 80-748001-30 (undetermined serotype), 7-72303C, 80-748001-28, 7-72303M (undetermined serotype), 7-72303P and 7-723033MC/MT from Dr I. Mehlman (FDA, Washington, DC); strain D6657, CDC 77-002524 from Dr R. Weaver (CDC, Atlanta, GA).

Mice. Six to eight-week-old male and female random-bred specific pathogen-free ICR-TRU mice, weighing 22–25 g were used in all experiments.

Pathogenicity test. Bacteria from an overnight culture in trypticase soy broth at 26°C were inoculated intravenously into mice in 0.2 ml of 0.15M NaCl solution. Growth of bacteria was followed in spleen, liver, and lungs after intravenous (i.v.) inoculation of 10³ bacteria. Groups of 3–5 animals were killed by cervical dislocation at $\frac{1}{2}$, 3, 6, and 12 h, and 1, 2, 5, and 10 days after inoculation. The organs were removed aseptically and homogenised separately in sterile saline as described by Collins (1970); 0.1 ml volumes of serial tenfold dilutions in saline were surface plated on TSA. The LD₅₀ was determined on groups of five mice after intravenous challenge, by the method of Reed and Muench (1938).

Culture media and test for calcium requirement. The calcium-containing media used were: Trypticase-soy Agar (TSA) and Trypticase-soy Broth (TSB) from BBL, Cockeysville, MD (calcium 40 mg/L, 0.4 mM; magnesium 20 mg/L, 0.1 mM). The three calcium-free media were (1) *Gelysate medium*: Gelysate Peptone 30 g (calcium and magnesium-free peptone, BBL), Agar Noble (Difco) 20 g, sodium gluconate 4.4 g, MgCl₂·6H₂O 4 g (0.02M final), double-distilled water 100 ml. (2) *Gelysate-oxalate medium*: the same medium supplemented with 80 ml of 0.25M sodium oxalate. (3) *Bactopeptone-oxalate medium*: Bactopeptone (Difco) 30 g, Agar Noble 20 g, MgCl₂·6H₂O 4 g (0.02M final), 0.25M sodium oxalate 80 ml, 1M glucose 10 ml, double-distilled water 1000 ml. As controls, these three calcium-free media were supplemented with 400 mg of CaCl₂ (4mM final).

The calcium-requirement tests, designed to assess the proportion of calcium-requiring colonies, were usually done on the gelysate-oxalate medium. Bacterial suspensions were made in double-distilled water and 0.1 ml of 10⁵ or 10⁶ dilutions was surface inoculated. After incubation for 24 h at 37°C, the large colonies were counted and the ratio of large to small colonies was assessed from the total number of colonies obtained after incubation at 26°C on the same medium.

The fluctuation test was done according to a protocol derived from that of Luria and Delbrück (1943). Two flasks containing 25 ml of TSB were inoculated with 10³ bacteria/ml.

FIG. 1.—**A.** Strain WA, after 36 h at 37°C on Gelysate medium, illustrating typical dissociation of colony size (arrowed), of highly virulent strains. **B.** Avirulent calcium non-requiring strain LOX-6, isolated from strain WA on the same medium at 37°C, showing uniform colony size.

One flask was immediately subdivided into twenty 1-ml portions that were each incubated overnight at 26°C. The second flask was incubated in the same conditions, then divided into twenty 1-ml portions. From each portion, tenfold dilutions in saline were made and 0.1 ml of each dilution was inoculated onto medium without calcium. Half of the plates were incubated at 26°C and the other half at 37°C and colony counting was done in quadruplicate. The percentage of calcium non-requiring colonies was determined by comparing the number of large colonies at 37°C with the total number of colonies at 26°C.

Sensitivity to the bactericidal effect of fresh mouse serum was determined by incubating 10^4 bacteria/ml in PBS containing 20% of either fresh mouse serum or mouse serum heated for 30 min at 56°C. The surviving bacteria were counted after 1 and 4 h in serum by plating on TSA.

RESULTS

Variation in colony size of Y. enterocolitica grown on calcium-free media

Incubation of 26 wild strains of *Y. enterocolitica* at 26° and 37°C on TSA and on the three calcium-free media supplemented with 4mM calcium, did not result in any differences in colony size. However, when grown on these same three calcium-free media, some strains formed colonies of two sizes, but only after incubation at 37°C. In such cases, most colonies were 0.5 mm after incubation for 36 h at 37°C (fig. 1A). The smaller number of large colonies (diameter 1–2 mm) appeared after incubation for 24 h. The difference in colony size was never apparent at 26°C on the same media without calcium (fig. 1B).

Eight strains (WA, BR, TFW 6331, 7-72303-C, E44, E2383, E2397, E2574), producing large and small colonies, were highly virulent (i.v. LD₅₀ < 10^3 bacteria per mouse) in that they caused systemic infections characterised by a rapid invasion of all organs with the formation of abscesses. The 18 strains not showing any colony dissociation at 26°C or 37°C were avirulent (i.v. LD₅₀ > 10^7 bacteria per mouse).

FIG. 2.—Growth of *Y. enterocolitica* strain WA and calcium non-requiring avirulent mutants LOX-6 and WA-LA in organs after intravenous inoculation of 10^3 bacteria per mouse.

Heterogeneity in the size of colonies growing on calcium-deficient media was evident only when the concentration of magnesium was 0.02M.

Virulence of large calcium non-requiring variants formed by mouse-virulent strains

The virulence of the large-colony variants formed when virulent strains were grown on medium without calcium at 37°C, was examined in mice by determining the LD₅₀. Three large colonies of each strain were separately subcultured on TSB and incubated overnight at 26°C. All had an LD₅₀ of $> 10^7$ bacteria per mouse. In contrast, all the tiny calcium-requiring colonies from the same plates, when similarly examined, had an LD₅₀ of $< 10^3$ bacteria per mouse.

The kinetics of growth in the organs of mice of the two kinds of colonial variants from the WA strain were examined after intravenous injection of 10^3 bacteria. After an initial destruction of more than 90% of bacteria in the liver, the parent WA strain gave rise to a very rapid systemic infection with early formation of abscesses in the liver, spleen, and lungs (fig. 2). The calcium-requiring variant grew with the same rapidity in organs (data not shown). In contrast, two calcium non-requiring variants, WA-LA and LOX-6, were almost completely eradicated in all organs within 48 h (fig. 2). It was obvious that small inocula of the avirulent mutants could not multiply in organs, whereas the wild strain grew progressively with a generation time of about 30 min during the first 12–24 h of infection in the spleen. After i.v. injection of 10^6 cells or variant LOX-6, the same profile of bacterial destruction in organs was observed (data not shown), and it was not possible to detect bacteria after 2–3 days. Moreover, abscesses were not visible in organs after this high challenge with LOX-6. These results were therefore similar to those from avirulent wild strains (Carter *et al.*, 1973).

The rapid decrease in the number of avirulent bacteria in the different organs was not related to sensitivity of the avirulent variants to the bactericidal effect of fresh mouse serum. None of the strains was destroyed by incubation *in vitro* for 1 h or 4 h with fresh or heated mouse serum.

Stability of calcium non-requiring variant after passage in vivo

When the eight virulent wild strains of *Y. enterocolitica* were incubated on medium without calcium at 37°C, the majority of colonies were slow growing and calcium dependent, but a variable, proportion (0.5–6%), of large calcium non-requiring colonies was observed. The same proportion of large colonies was obtained when the tests were performed with micro-organisms grown in TSB at 26°C or 37°C. Indeed, the same result was obtained even when organisms were plated directly from heavily infected homogenised mouse tissues that were highly diluted in double-distilled water to cause depletion of calcium.

Continuous cloning of the calcium-requiring colonies in TSB at 26° or 37°C gave the same proportion of small to large colonies after plating on calcium-depleted medium. In contrast the calcium non-requiring colonies remained stable after recloning by the same technique. A reversal to small-colony morphology was never observed after plating as many as 10^5 large colonies on Gelysate-oxalate medium at 37°C.

The factor responsible for inducing avirulent large colonies at 37°C

To distinguish between induction of and selection for avirulent large colonies on calcium-free medium (Gelysate-oxalate medium), an experiment was performed with

TABLE

Fluctuation test at 37°C on Gelysate-oxalate medium without calcium with the virulent strain WA

Sample no.	Number of large colonies in group	
	A	B
1	6	9
2	18	8
3	17	14
4	11	17
5	9	9
6	12	11
7	16	9
8	16	11
9	7	14
10	15	15
11	14	21
12	17	9
13	18	22
14	14	18
15	16	20
16	7	14
17	14	15
18	11	14
19	16	16
20	18	19
Mean $\pm$ SD	13.6 $\pm$ 3.90	14.2 $\pm$ 4.34

Group A: secondary separation of the tryptone-soya broth (TSB) samples after incubation for 16 h at 26°C. Group B: immediate separation of the TSB samples after inoculation of bacteria.

* $p > 0.05$ by Student's t test

the WA strain by the fluctuation test of Luria and Delbrück (1943). The table shows that there was no statistically significant difference between the number of large-colony variants in samples that were subdivided immediately after the inoculation of bacteria (group B) and those that were subdivided 16 h after culture (group A). This experiment was repeated twice with the same strain and gave the same result. These results indicate, therefore, that calcium non-requiring colonies are induced at 37°C by lack of calcium and do not appear spontaneously.

DISCUSSION

This work demonstrates that in *Y. enterocolitica* the association between calcium requirement at 37°C and virulence, previously described for three virulent strains (Carter *et al.*, 1980; Gemski *et al.*, 1980) is probably a general phenomenon. None of the 18 avirulent strains displayed such a calcium requirement, whereas all of the eight virulent strains did. There exists, therefore, a striking similarity between *Y. enterocolitica* and *Y. pestis*, in that highly virulent strains of both species have a magnesium-dependent requirement for calcium (Higuchi *et al.*, 1959). For *Y. pestis*, a rapid loss of virulence on medium without calcium with a mutation rate to avirulence of 0.1–1% has been previously reported (Higuchi and Smith, 1961; Brubaker and Surgalla, 1964). We observed an almost identical rate of mutation to avirulence for virulent strains of *Y. enterocolitica*. It might be significant, in terms of expression of virulence, that the concentration of calcium and magnesium ions in the cytoplasm of

macrophages, as in any other cell, is very similar to the concentration of these ions in culture media used to obtain avirulent mutants *Yersinia*. The demonstration of growth inhibition of *Y. pestis* at 37°C in medium without calcium (Carter *et al.*, 1980), is apparently contradictory to the demonstration of multiplication of this micro-organism inside macrophages at 37°C (Janssen *et al.*, 1963; Janssen and Surgalla, 1969). In this context it may be important that the VW antigens of *Y. pestis*, described by Burrows and Bacon (1956), are produced maximally at 37°C in medium without calcium. Carter *et al.*, (1980) reported that, in medium without calcium at 37°C, *Y. enterocolitica* strain WA produced VW antigens identical to those produced by virulent strains of *Y. pestis* (Burrows and Bacon, 1956) and *Y. pseudotuberculosis* (Burrows and Bacon, 1960). These antigens were not produced by the avirulent calcium non-requiring strains obtained after subculture on medium without calcium at 37°C (Carter *et al.*, 1980). However, it has not yet been established whether *Y. enterocolitica* can survive intracellularly.

The association between the virulence of *Y. enterocolitica* and the presence of a plasmid has been established by Zink *et al.*, (1978) and Portnoy, Moseley and Falkow, (1981), who demonstrated the presence of a plasmid in the invasive strains of this organism. Gemski *et al.*, (1980) related calcium requirement and virulence to a plasmid in three highly virulent strains of *Y. enterocolitica*, including strain WA. Treatment with ethidium bromide caused the three calcium-requiring strains to lose their plasmid and become avirulent and calcium non-requiring. This relationship between calcium requirement and the loss of a plasmid could explain the surprisingly high mutation rate to avirulence observed with the virulent strains of *Yersinia*. This interesting finding could be related to our result in the fluctuation test in which there was no significant difference between the proportion of non-requiring colonies in the samples that were subdivided immediately or 16 h after the inoculation of bacteria. According to the interpretation of Luria and Delbrück (1943), these results demonstrate that the emergence of calcium non-requiring colonies is induced at 37°C by the lack of calcium rather than being the result of a spontaneous mutation. Moreover, this result correlates with the fact that the same proportion of non-requiring colonies could be isolated from the organs of heavily infected mice. Because these calcium non-requiring bacteria are rapidly destroyed in the tissues (fig. 2), the observation that they could be isolated from abscesses, where most bacteria are extracellular, argues in favour of an in-vitro induction. It is possible that the lack of calcium at 37°C would trigger a rapid loss of a plasmid coding for one or several antigens associated with virulence.

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