INFECTION AND IMMUNITY, Oct. 1989, p. 3234–3236
0019-9567/89/103234-03$02.00/0

Vol. 57, No. 10

Virulence, Persistence, and Immunogenicity of *Yersinia enterocolitica* O:8 *aroA* Mutants

FRANCES BOWE,[1,2] PEADAR O'GAORA,[1,2] DUNCAN MASKELL,[1]† MARY CAFFERKEY,[2] AND GORDON DOUGAN[1]*

Department of Molecular Biology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, United Kingdom,[1] and Moyne Institute, Trinity College, Dublin 2, Ireland[2]

Received 1 May 1989/Accepted 30 June 1989

The virulent *Yersinia enterocolitica* strain 8081 killed BALB/c mice within 5 days of oral infection with a 50% lethal dose of $\log_{10}$ 7.1, whereas an *aroA* mutant of 8081, YAM.1, and the plasmidless variant 8081c failed to kill mice. Unlike 8081, YAM.1 and 8081c did not persist or grow in the Peyer's patches, mesenteric lymph nodes, livers, or spleens of mice. Mice immunized orally with single doses of live YAM.1 were poorly protected against a lethal 8081 challenge, whereas mice immunized with three doses of YAM.1 were moderately well protected.

Yersinia enterocolitica causes a spectrum of diseases ranging from those confined to the intestinal tract to fatal septicemic disease (1, 2, 6, 11, 14). Serotypes isolated in Europe usually produce self-limiting gastroenteritis, whereas American strains, especially serotype O:8, are more likely to cause systemic infections (3, 4, 8, 9). *Y. enterocolitica* O:8 strains are also virulent for mice. After being inoculated orally with these strains, mice experience systemic infections involving such organs of the reticuloendothelial system as Peyer's patches, mesenteric lymph nodes, livers, and spleens (5). Virulent *Y. enterocolitica* strains harbor a plasmid which encodes a number of virulence-associated determinants (16); loss of this plasmid leads to attenuation in mice (12, 19). *Salmonella* spp., including *Salmonella typhimurium*, also cause invasive infections in mice following oral challenge (7). Virulent salmonellae can be attenuated by the introduction of stable mutations in genes in the aromatic biosynthetic pathway (10). Such mutants (*aro* mutants) require aromatic compounds, including the aromatic amino acids *para*-aminobenzoic acid and dihydroxybenzoate, for growth. Since some of these compounds are in short supply in vivo, the bacteria are capable of only limited replication before being cleared from the host (13, 15). Extensive studies on the immunological properties of *Salmonella aroA* mutants have shown them to be excellent oral vaccines (10, 13, 15). Little information is available on whether *aro* lesions can attenuate bacteria other than *Salmonella* spp. *aro* mutants of *Yersinia* spp., if attenuated, would be useful immunological tools for the study of immune responses to *Yersinia* spp. and for the development of *Yersinia* species-specific vaccines. We have recently cloned and sequenced the *aroA* gene of 8081, a *Y. enterocolitica* O:8 strain, and used the cloned gene to construct an *aroA* mutant of this strain referred to as YAM.1 (P. O'Gaora, D. Maskell, M. Cafferkey, D. Coleman, and G. Dougan, Gene, in press). In this report, we describe the behavior of the *Y. enterocolitica* O:8 *aroA* YAM.1 strain in vivo and compare it with the behavior of a plasmidless variant of the same strain.

Virulence and persistence of *Y. enterocolitica* variants in BALB/c mice. 8081 is a mouse-virulent *Y. enterocolitica* O:8 strain. 8081c is a plasmidless variant of 8081. Both strains were gifts from Mikael Skurnik, University of Umea, Umea, Sweden. YAM.1 is a *Y. enterocolitica aroA* mutant which harbors a 1.2-kilobase-pair DNA sequence encoding kanamycin resistance inserted into the *aroA* gene and which thus depends on aromatic compounds for growth in vitro. All strains exhibited normal lipopolysaccharide profiles when analyzed on polyacrylamide gels by the method of Tsai and Frasch (20). Bacteria were routinely cultured in tryptic soy broth or on tryptic soy agar (Difco, Scunthorpe, United Kingdom). Cefsulodin irgasan novobiocin (18) agar (Difco) was used as a selective medium for *Y. enterocolitica*.

BALB/c male mice, aged approximately 8 weeks and bred at Wellcome Research Laboratories, were used throughout. Oral inoculations and the determination of in vivo bacterial viable counts were carried out as described previously (18). Gastric acidity was neutralized before all oral inoculations by the oral administration of 0.2 ml of 7.5% sodium bicarbonate. The 50% lethal doses (LD_{50}s) were calculated by the method of Reed and Muench (17).

Y. enterocolitica strains were tested for virulence after being administered orally to BALB/c mice. 8081 was virulent, with an LD_{50} of $\log_{10}$ 7.1. On the other hand, 8081c and YAM.1 were nonlethal, even after the administration of more than $\log_{10}$ 10 organisms. Thus, YAM.1 and 8081c were highly attenuated. Experiments were conducted to determine the growth kinetics of the *Yersinia* strains in vivo. Oral infection of 40 mice with doses of $\log_{10}$ 10 wild-type 8081 organisms resulted in their deaths within 5 days, with concomitant high levels of bacteria in their reticuloendothelial systems (Fig. 1). However, oral infection with YAM.1 resulted in no deaths. Peak counts were obtained on day 1 postinfection, with the highest counts ($\log_{10}$ 2.82) found in mesenteric lymph nodes (Fig. 2A). Bacteria were also detected in Peyer's patches, but only two of four livers and no spleens sampled were infected. Bacteria were undetectable in livers and mesenteric lymph nodes by day 5 postinfection. Organisms were detected in one Peyer's patch on day 5 but were undetectable in all Peyer's patches on subsequent days. Similar results were obtained with two other similar *Yersinia aroA* mutants, YAM.2 and YAM.3 (results not shown).

For comparative purposes, the persistence of 8081c was

* Corresponding author.
† Present address: Molecular Infectious Diseases Group, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom.

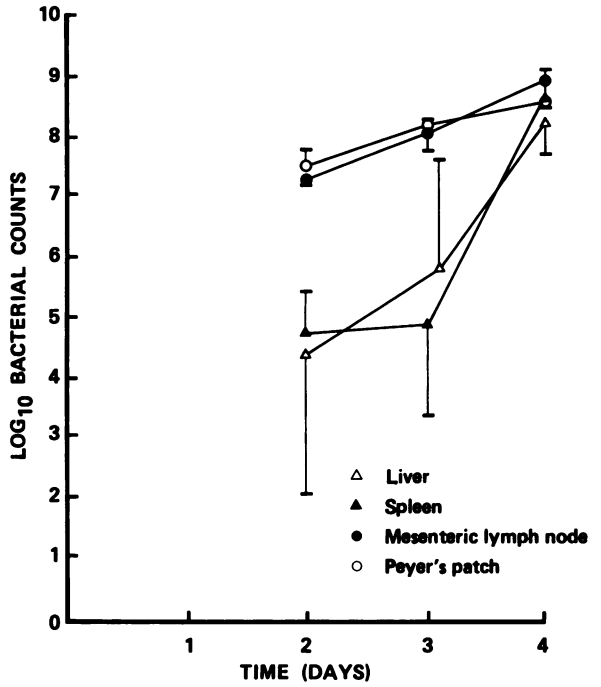

FIG. 1. Colonization of the organs of BALB/c mice following oral administration of strain 8081. Each point represents the geometric mean ± 2 standard errors for four mice. Mice which had not already been sacrificed were dead by day 5 postinfection. Controls remained healthy.

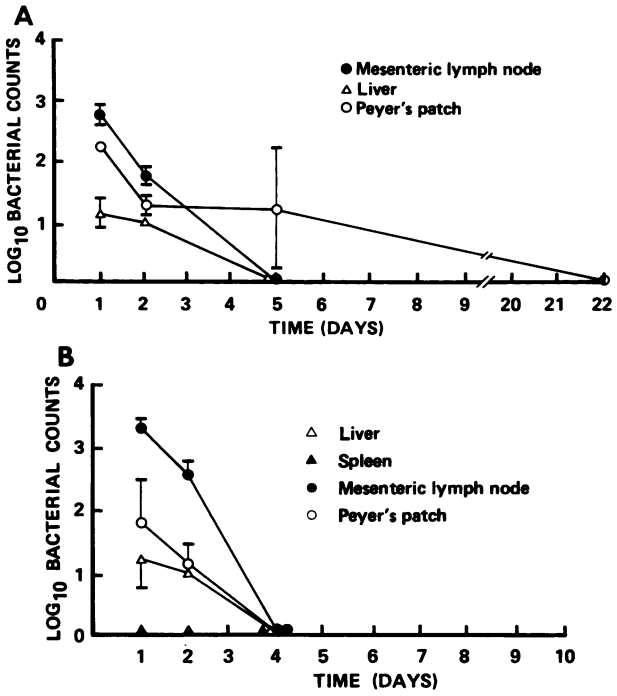

FIG. 2. Colonization of the organs of BALB/c mice following oral administration of strains YAM.1 (A) and 8081c (B). Each point represents the geometric mean ± 2 standard errors for four mice.

monitored in a similar manner. The persistence of 8081c in all organs tested was strikingly similar to that of YAM.1 (Fig. 2B). Again, oral infection resulted in no deaths. On day 1 postinfection, bacteria were detected at low levels in Peyer's patches and mesenteric lymph nodes, while only two livers and no spleens sampled were infected. Counts in all organs decreased with time and, by day 4 postinfection, bacteria could not be detected in any of the organs. 8081c was completely cleared from the intestines of mice within 12 days of infection.

Although wild-type *S. typhimurium* and *Y. enterocolitica* strains exhibit similar virulence for and growth kinetics in mice, *aroA* mutants of these organisms clearly differ in their growth kinetics in vivo. YAM.1 could establish only a short-lived colonization of the reticuloendothelial system, similar to that detected with 8081c. *S. typhimurium aroA* mutants, when administered orally to BALB/c mice, invade from the gut and establish a self-limiting infection involving colonization of Peyer's patches, mesenteric lymph nodes, livers, and spleens. Up to $\log_{10}$ 4 organisms can be detected in different tissues several days after challenge, and the bacteria are not completely cleared for several weeks (13). These data suggest that there may be fundamental differences between the mechanisms of in vivo growth and persistence used by *Yersinia* and *Salmonella* spp. Yersiniae may be much more sensitive than salmonellae to the bacterial clearance mechanisms operating in vivo, relying on rapid and overwhelming growth to kill the mice before clearance can be effective. Alternatively, there may be subtle differences in the abilities of salmonellae and yersiniae to metabolize aromatic compounds.

Immunogenicity of orally administered attenuated variants. As a preliminary experiment to assess the immunogenicity of YAM.1, all mice surviving the earlier LD_{50} determination were challenged with single $\log_{10}$ 9.55 doses of virulent 8081. A dose-response effect was observed (Table 1), showing that the mean time to death increased as the immunizing dose of YAM.1 increased. Immunized animals died between days 8 and 17, whereas controls were all dead by day 8. Two immunized animals survived the challenge. While these results indicate that immunization delayed death from a virulent infection, most of the mice still died. Accordingly, we investigated the immunizing properties of multiple oral doses of YAM.1. Forty BALB/c mice were immunized orally with $\log_{10}$ 9.96, 10.75, and 10.52 doses of YAM.1 at 3-day intervals. A control group of mice were dosed on each occasion with phosphate-buffered saline. On day 28 after the final immunization, the mice were challenged orally with a maximum $\log_{10}$ 8.45 dose of 8081 to determine the LD_{50}. The LD_{50} value obtained for the control group treated with phosphate-buffered saline was $\log_{10}$ 6.88. Only four animals in the immunized group died, giving an LD_{50} of more than $\log_{10}$ 8.55 (Table 2). The survival rate for immunized animals

TABLE 1. Results of oral challenge with 8081 of mice surviving LD_{50} experiment with YAM.1[a]

Immunizing dose ($\log_{10}$)	No. of deaths[b]/total no. of mice	Mean time to death (days) ± SE
10.32	7/8	12.43 ± 1.27
9.32	7/8	13.43 ± 2.44
8.32	10/10	9.80 ± 0.42
7.32	10/10	9.20 ± 0.79
0	5/5	8.00 ± 0.00

[a] Mice surviving an oral LD_{50} challenge with YAM.1 were challenged orally 28 days later with a $\log_{10}$ 9.55 dose of 8081.
[b] Deaths were recorded for 28 days after challenge with strain 8081.

TABLE 2. Survival of mice immunized with three doses of strain YAM.1 following oral challenge with 8081[a]

8081 challenge dose (log_{10})	No. of deaths[b]/total no. of mice	
	Immunized	Control
8.45	1/10	10/10
7.45	2/10	4/10
6.45	1/10	2/10
5.45	0/9	0/10

[a] Mice were immunized on days 0, 3, and 6 with log_{10} 9.96, 10.75, and 10.52 doses of YAM.1, respectively, and challenged on day 33 with strain 8081.
[b] Deaths were recorded for 28 days after challenge with 8081.

following challenge with a log_{10} 8.45 dose of 8081 was 90%. This contrasts strongly with the outcome for unimmunized mice, of which there were no survivors at this challenge level. Thus, BALB/c mice orally immunized with three doses of YAM.1 were clearly protected against virulent oral challenge with the *Y. enterocolitica* parent strain.

These results show that *Y. enterocolitica* O:8 *aroA* mutants are less effective oral vaccines than are *S. typhimurium aroA* mutants: several doses of the *Yersinia aroA* mutants are required for measurable protection, whereas *Salmonella aroA* mutants confer protection after a single dose (15). This may result from the poor persistence of the *Yersinia* mutants. We are currently assessing the immune responses in the protected animals to examine in detail the mechanisms of protection. It will be interesting to see how *Y. enterocolitica aroA* mutants behave in other infection models and to see if *aroA* mutants of other *Yersinia* species are also attenuated.

It is possible that *aro* and other metabolic lesions may provide a common route by which to attenuate a variety of bacterial pathogens. We have recently shown that *aroA* mutants of *Bordetella pertussis* are highly attenuated and immunogenic in a mouse aerosol challenge system (M. Roberts, D. Maskell, P. Novotny, and G. Dougan, submitted for publication). Thus it may soon be possible to develop a range of rationally attenuated bacteria for use as immunological tools and experimental vaccines.

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