Immunological analysis of the plasmid-encoded proteins from the highly pathogenic *Yersinia enterocolitica* serogroup O8 and the less pathogenic serogroup O3

M. NAUMANN*, C. HANSKI and A. GRÜTZKAU†

Klinikum Steglitz, Abteilung Innere Medizin mit Schwerpunkt Gastroenterologie, der Freien Universität Berlin, Berlin, Germany

Summary. The plasmid-encoded proteins of the pathogenic *Yersinia enterocolitica* serogroups O3 and O8 were analysed with respect to their immunological relationship. Common epitopes on yersinia outer-membrane proteins (YOPs) and released proteins (RPs) were recognised by orally-induced antisera against living bacteria and by monospecific antisera induced against single RPs of *Y. enterocolitica* serogroup O8. A major difference between the pathogenic serogroups O3 and O8 was YOP42, which was detected only in the outer membrane of the highly pathogenic O8⁺ bacteria. The YOP42 may be responsible for the greater virulence of serogroup O8 bacteria.

Introduction

Pathogenic strains of *Yersinia enterocolitica* possess a 42–46-MDa plasmid which is sensitive to environmental conditions.¹ At low calcium ion concentration and temperatures around 37°C this plasmid encodes several gene products responsible for virulence. These include proteins released (RPs) into the culture supernate.² Some of these are structurally related to several temperature-inducible yersinia outer-membrane proteins (YOPs) which accumulate in the outer membrane under appropriate incubation conditions and are produced by yersiniae growing *in vivo*.³

Although they share similar plasmids, strains of serogroup O8 are more virulent than those of serogroup O3 and can cause invasive disease.⁴ Thus, following oral infection of mice, serogroup O8 was able to traverse the mesenteric lymph nodes and cause systemic infection with persistence in the kidneys, liver, spleen, lungs and the bloodstream.⁵ Although virulence has been shown to correlate with the presence of plasmid-encoded proteins in the pathogenic strains,⁶ the factors that are responsible for the survival of the highly pathogenic serogroup O8 are still unknown. The purpose of this study was to obtain further information about the antigenicity of the plasmid-encoded proteins within different pathogenic serogroups. To evaluate more precisely the extent to which these proteins share common antigenic determinants,

they were analysed with antisera to proteins induced under different conditions.

Materials and methods

Bacterial strains and growth conditions

A plasmid-bearing *Y. enterocolitica* strain (NCTC 10938) of serogroup O8, denoted O8⁺, and an isogenic plasmid-free strain (NCTC 10598), denoted O8⁻, were obtained from the National Collection of Type Cultures, Colindale Avenue, London. A plasmid-bearing strain (54/2b) of serogroup O3, denoted O3⁺, was obtained from Professor Hellmann, Institut für Veterinärmedizin, Berlin. A plasmid-free derivative of strain 54/2B, denoted O3⁻, was isolated on magnesium-oxalate agar after the plasmid-bearing strain had been plated and incubated at 37°C.⁷ The bacteria were grown overnight in brain-heart infusion broth (BHB) at 24°C. This pre-culture was then diluted 1 in 15 with fresh BHB and incubated with shaking at 37°C for 2 h. For optimal expression of YOPs and RPs, the medium was supplemented with 10 mM EDTA and incubation was continued for a further 2 h.

Isolation of YOPs and RPs

Outer membranes were isolated according to the method of Bölin *et al.*⁸ Total RPs were purified by the method of Heesemann *et al.*,⁹ and additionally dialysed against phosphate-buffered saline (PBS).

Production of antisera

Rabbit antisera against living bacteria of the O8⁺

Received 11 Feb. 1992; accepted 29 June 1992.

*Present address: Max-Planck-Institut für Molekulare Genetik, Otto-Warburg-Laboratorium, Ihnestr. 73, 1000 Berlin-Dahlem, Germany.

† Present address: Schering AG, Biochemische Pharmakologie, Müllerstr. 170–178, 1000 Berlin 65, Germany.

strain were raised and pre-adsorbed according to procedures described previously.^{4,10}

For the induction of the monospecific antisera against single RPs of the O8⁺ strain, the proteins were separated according to Forsberg *et al.*¹¹ by excising the KCl-stained protein band from the SDS-PAGE gel as described by Hager and Burgess.¹² The SDS-PAGE gel pieces were homogenised in PBS by passing them through a syringe and needle. The homogenised gel containing *c.* 100 µg of protein was used for the first injection. For two booster injections, which followed at 2-week intervals, *c.* 25 µg of protein was injected.

Immunoblotting

The YOPs and RPs were separated in SDS-acrylamide 12% gels according to the method of Laemmli¹³ and transferred to a PVDF Immobilon membrane (Millipore, Bedford, USA) as described previously.¹⁴ Immunostaining was performed as recommended by the supplier of Immunogold (Janssen Biotech, Olen, Belgium). Briefly, the membranes were incubated overnight with orally-induced antisera at a dilution of 1 in 500, and with the anti-RP antisera at a dilution of 1 in 64000. After washing, the membranes were incubated overnight with goat anti-rabbit IgG coupled with colloidal gold at a dilution of 1 in 100. For silver enhancement the filters were incubated for 15–30 min in a mixture of equal volumes of Initiator and Enhancer solution (IntenseBL, Janssen Biotech).

Results

Identification of plasmid-encoded proteins in *Y. enterocolitica* serogroups

YOPs and RPs from serogroups O3 and O8 were identified by SDS-PAGE and silver staining (fig. 1). In outer-membrane preparations, several YOPs were identified in both serogroups (tracks 3 and 6) which were absent from the outer membranes of plasmid-free bacteria (tracks 1 and 7). Some of the RPs and some of the YOPs exhibit corresponding mol. wts in the SDS-PAGE gels (compare tracks 2, 3, 4 and 5).

Immunological analysis of YOPs and RPs in *Y. enterocolitica* serogroups

An orally-induced antiserum against plasmid-bearing bacteria of the O8⁺ strain was used for the immunological analysis of plasmid-encoded proteins. Antibodies against chromosome-encoded antigens were exhaustively pre-adsorbed by incubation of the antiserum with excessive amounts of intact O8⁻ bacteria. During this procedure, those antibodies which had free access to epitopes on the bacterial surface were removed from the antiserum. The pre-adsorbed antiserum recognised several proteins in the outer membrane of the O8⁺ strain (fig. 2a, track 2) which

Fig. 1. Expression of YOPs and RPs in different *Y. enterocolitica* serogroups. Proteins were separated by SDS-PAGE in a gradient of acrylamide (8–18%) and silver stained: track 1, outer membrane of O8⁻ strain; 2 and 3, outer membrane of O8⁺ strains grown in the presence or absence of CaCl₂; and 3, 4 and 5, RPs of strains O8⁺ and O3⁺; 6 and 7, outer membrane of strains O3⁺ and O3⁻. Proteins are indicated by arrows.

were not present in the outer membrane of the O8⁻ strain (fig. 2a, track 1). Moreover, the antiserum reacted with most of the RPs (fig. 2a, track 3). The RPs were not produced by plasmid-free bacteria (fig. 2a, track 4). The apparent mol. wts of the recognised proteins are indicated and listed in the table. The same antiserum against O8⁺ bacteria recognised several YOPs and RPs (fig. 2b, tracks 2 and 3) of the O3⁺ strain which were not present in the O3⁻ strain (fig. 2b, track 1).

For further investigations of the serological relationship between YOPs and RPs, four monospecific antisera generated against the main RPs of the O8⁺ strain were used in the immunoblot analysis. Each of these antisera detected a YOP in the outer-membrane preparations of the O8⁺ strain, with corresponding mol. wts (56, 47, 34 and 24 kDa) (fig. 3, tracks 1, 3, 5 and 7). In outer-membrane preparations of the O3⁺ strain, the same antisera also recognised YOPs of the same apparent mol. wts as those recognised in the O8⁺ strain (tracks 2, 4 and 6), with the exception that the anti-RP56 antiserum reacted with a 44-kDa YOP (track 8).

Fig. 2. **a**, Immunoblot of plasmid-encoded proteins from *Y. enterocolitica* strains of serogroup O8. Development was performed with an orally-induced antiserum against O8⁺ bacteria in a 1 in 500 dilution: tracks 1 and 2, outer membranes of the plasmid-free strain O8⁻; and the plasmid-bearing strain O8⁺; 3, RPs of strain O8⁺; 4, RP preparation of strain O8⁻. **b**, Tracks 1 and 2, outer membranes of the plasmid-free strain O3⁻ and the plasmid-bearing strain O3⁺; 3, RPs of strain O3⁺. Arrows point to YOPs and RPs recognised by the antiserum.

Table. Relative M_r (kDa) of plasmid-encoded proteins from *Y. enterocolitica* O3 and O8 serogroups determined by SDS-PAGE

Serogroup O8		Serogroup O3	
YOPs	RPS	YOPs	RPs
180 (P1)		210 (P1)	
79	79	79	79
		69	69
56	56		
		51*	
47	47	47	47
45*			
44*		44	
42			39
	38		
			35
34	34	34	34
	32		32
	28		28
26		26	
24	24	24	24

* Subunits of protein P1.

Fig. 3. Immunoblot of outer membranes from *Y. enterocolitica* strains of serogroups O8 (tracks 1, 3, 5 and 7) and O3 (2, 4, 6 and 8), developed with monospecific antisera to RPs of strain O8⁺. Monospecific antisera generated against RP24 (tracks 1 and 2), RP34 (3 and 4), RP47 (5 and 6) and RP56 (7 and 8) were applied at a dilution of 1 in 64000. Arrows indicated YOPs recognised by each monospecific antiserum.

Discussion

This study showed that the orally-induced pre-adsorbed antiserum and the monospecific antisera, which were raised against RPs from the O8⁺ strain, exhibited high cross-reactivity against YOPs and RPs

within both serogroups investigated. In agreement with earlier studies on *Y. pseudotuberculosis*,¹¹ these results indicate the presence of highly conserved antigenic domains within the plasmid-encoded proteins of pathogenic yersiniae.

In contrast to YOPs, the RPs were not detected in

infected Peyer's patches,¹⁰ which suggests that they are not required for pathogenesis of *Y. enterocolitica* infection. One of the major differences between the two serogroups, which could be important for virulence, was the presence of YOP42 in the highly pathogenic O8 serogroup. Also, YOP56 in the O8⁺ bacteria, although immunologically related to YOP44 from the O3⁺ bacteria, may exert an important virulence function in the O8 serogroup.

The proteins of 45 and 44 kDa in serogroup O8, and 51 kDa in serogroup O3 represent the subunits of

protein P1 (data not shown). Although the subunit structure of the outer-membrane protein P1 varies between *Y. enterocolitica* serogroups, P1 has no relevance for pathogenicity.¹⁵ Possibly individual YOPs, such as YOP42 and the additional YOPs determined *in vivo*,^{10,16} possess important functions for persistence in the host tissue.

This work was supported by grant Ha 152/1-2 from the Deutsche Forschungsgemeinschaft and by the Maria-Sonnenfeld-Gedächtnisstiftung.

References

1. Ben-Gurion R, Schafferman A. Essential virulence determinants of different *Yersinia* species are carried on a common plasmid. *Plasmid* 1981; **5**: 183-187.
2. Heesemann J, Gross U, Schmidt N, Laufs R. Immunochemical analysis of plasmid-encoded proteins released by enteropathogenic *Yersinia* sp. grown in calcium-deficient media. *Infect Immun* 1986; **54**: 561-567.
3. Bölin I, Portnoy DA, Wolf-Watz H. Expression of the temperature-inducible outer membrane proteins of yersiniae. *Infect Immun* 1985; **48**: 234-240.
4. Hanski C, Naumann M, Hahn H, Riecken EO. Determinants of invasion and survival of *Yersinia enterocolitica* in intestinal tissue: an in-vivo study. *Med Microbiol Immunol* 1989; **178**: 289-296.
5. Falcao DP, Shimizu MT, Trabulsi LR. Kinetics of infection induced by *Yersinia*. *Curr Microbiol* 1984; **11**: 303-308.
6. Cornelis G, Laroche Y, Balligand G, Sory M-P, Wauters G. *Yersinia enterocolitica*, a primary model for bacterial invasiveness. *Rev Infect Dis* 1987; **9**: 64-87.
7. Gemski P, Lazere JR, Casey T. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. *Infect Immun* 1980; **27**: 682-685.
8. Bölin I, Norlander L, Wolf-Watz H. Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is associated with the virulence plasmid. *Infect Immun* 1982; **37**: 506-512.
9. Heesemann J, Algermissen B, Laufs R. Genetically manipulated virulence of *Yersinia enterocolitica*. *Infect Immun* 1984; **46**: 105-110.
10. Naumann M, Hanski C, Riecken EO. Expression *in vivo* of additional plasmid-mediated proteins during intestinal infection with *Yersinia enterocolitica* serotype O8. *J Med Microbiol* 1991; **35**: 257-263.
11. Forsberg A, Bölin I, Norlander L, Wolf-Watz H. Molecular cloning and expression of calcium-regulated, plasmid coded proteins of *Y. pseudotuberculosis*. *Microb Pathog* 1987; **2**: 123-137.
12. Hager DA, Burgess RR. Elution of proteins from sodium dodecyl sulfate, polyacrylamide gels, removal of sodium dodecyl sulfate and renaturation of enzymatic activity: results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. *Anal Biochem* 1980; **109**: 76-86.
13. Laemmli UK. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680-685.
14. Naumann M, Hanski C, Friedrich B, Riecken EO. A rapid *in situ* expression test for virulence-associated proteins of *Yersinia enterocolitica*. *J Microbiol Methods* 1990; **11**: 83-93.
15. Rosqvist R, Skurnik M, Wolf-Watz H. Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. *Nature* 1988; **334**: 522-525.
16. Hanski C, Naumann M, Grützkau A *et al.* Humoral and cellular defense against intestinal murine infection with *Yersinia enterocolitica*. *Infect Immun* 1991; **59**: 1106-1111.