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Heat-stable serogroup-specific proteins of *Yersinia pseudotuberculosis*

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A library of mAbs to the species- and serogroup-specific epitopes of *Yersinia pseudotuberculosis* serogroups I–VI was developed. These mAbs recognized linear sequential protein epitopes, as shown by ELISA and immunoblotting. Using the mAbs, *Y. pseudotuberculosis* was found to produce serogroup-specific proteins, whose synthesis was dependent on cultivation temperature. These proteins appeared to be parts of heat-stable O-antigens prepared by heating *Y. pseudotuberculosis* serogroups I–VI at 100 °C for 2 h, and are responsible for the protein serotype specificity of these bacteria. The high specificity of serogroup- or species-specific mAbs obtained in ELISA suggests that they may be effective for serotyping of *Y. pseudotuberculosis* strains or differentiation from other pathogenic yersiniae.

INTRODUCTION

Yersinia pseudotuberculosis is classified into serogroups on the basis of its somatic heat-stable (O-) antigen (Knapp & Weber, 1982; Somov *et al.*, 1990; Ovodov *et al.*, 1992) or its endotoxin (Skurnik, 1999; Skurnik *et al.*, 2000), which represents a complex of LPS with outer-membrane proteins (OMPs) (Solov'eva *et al.*, 1990). The chemical structure and some immunobiological activities of LPSs, including the O-side chains of each *Y. pseudotuberculosis* serogroup or O-subgroup, have been studied (Knapp & Weber, 1982; Somov *et al.*, 1990; Ovodov *et al.*, 1992; Skurnik, 1999). However, the role of proteins in the immunoreactivity of O-antigens of *Y. pseudotuberculosis* is still not clearly defined. The immunological and structural diversity of protein antigens (OMPs, capsular antigens, H- or Vi-antigens, etc.) together with LPSs provides the basis for serological classification of the family *Enterobacteriaceae* (Frasch & Gotschlich, 1974; Zollinger & Mandrell, 1980; Ogasawara *et al.*, 1985; Perry & Fetherston, 1997; Pokrovsky & Posdeev, 1999; Nandi *et al.*, 2000; Feodorova *et al.*, 2001). *Y. pseudotuberculosis* is able to produce some species-specific proteins (Ogasawara *et al.*, 1985; Braithwaite *et al.*, 1993; Aksenov *et al.*, 1995; Nedashkovskaya *et al.*, 1995; Burgasova *et al.*, 1996; Drobkov *et al.*, 1996; Kulyashova *et al.*, 1997), but serogroup-specific proteins have not been reported.

In this study, several mAbs to serogroup- or species-specific protein epitopes of *Y. pseudotuberculosis* were characterized. The presence of heat-stable protein components in heat-stable O-antigens, bearing linear sequential epitopes complementary to serogroup-specific mAbs and providing serological specificity of *Y. pseudotuberculosis*, was shown. The application of ELISA based on mAbs for serotyping of *Y. pseudotuberculosis* and its differentiation from other pathogenic yersiniae is discussed.

Abbreviations: OMP, outer-membrane protein; PK, proteinase K.

METHODS

Bacterial strains. *Y. pseudotuberculosis* strains used in this study are listed in Table 1. In addition, 24 strains of other bacteria (eight *Yersinia pestis*, six *Yersinia enterocolitica*, two *Escherichia coli*, three *Proteus* strains, three *Shigella* strains and two *Salmonella* strains) were employed. All the strains except *Y. pestis* were grown on Hottinger agar, pH 7·2, at 37 °C for 48 h, or at 28 °C for 48 h for some experiments. *Y. pestis* strains were grown on Hottinger agar, pH 7·2, at 28 °C for 48 h, or for some experiments at 28 °C for 24 h followed by 37 °C for 24 h.

Production and purification of mAbs. The conventional fusion procedure using PEG was used, with minor modifications (Galfre *et al.*, 1977; Feodorova & Devdariani, 2000). Briefly, to prepare sensitized spleen cells, female BALB/c mice, 6–10 weeks old and weighing 18–20 g, were given three intraperitoneal doses of sodium merthiolate-killed whole cells of *Y. pseudotuberculosis* serogroup II at 2-week intervals, according to the following schedule. The first injection contained 2×10^4 microbial cells emulsified in complete Freund's adjuvant (Sigma), the second contained 3×10^4 and the third, 6×10^4 microbial cells. After the final boost, the mice were killed by inhalation of ether. Spleens were collected aseptically from the mice and pressed through a sterile 5 ml syringe into tissue culture medium, to produce a single-cell suspension. The cells were pelleted by centrifugation; erythrocytes were removed by hypotonic lysis with sterile 0·83 % NH_4Cl in distilled water. The spleen cells were then fused with the Sp2/0-Ag14 BALB/c myeloma cell line (Feodorova & Devdariani, 2000). Emerging clones were screened by ELISA. Selected hybridomas were cloned by limiting dilution and were grown in bulk. mAbs were purified by affinity chromatography as described previously (Feodorova & Devdariani, 2000) and used in all experiments. The class and subclass of the mAbs were determined by immunodiffusion (Feodorova & Devdariani, 2000) and found to be IgG1.

Table 1. *Y. pseudotuberculosis* strains used in this study

Strain	Description	Source*
CIP 55.85^T	Serogroup I reference strain	CIP
CIP 55.86	Serogroup II reference strain	CIP
CIP 55.87	Serogroup III reference strain	CIP
CIP 55.88	Serogroup V reference strain	CIP
CIP 55.89	Serogroup IV reference strain	CIP
110	Serogroup VI reference strain	IMC
603-73	Clinical isolate, Russia, 1973, serogroup I	IMC
10-B	Clinical isolate, Russia, 1966, serogroup I	IMC
15-73	Clinical isolate, Russia, 1973, serogroup I	IMC
79 (212 Lucas)	Isolated in 1966, serogroup I	HM
132-D	Clinical isolate, Russia, 1967, serogroup I	IMC
A17	Clinical isolate, Russia, 1977, serogroup I	IMC
50-73	Clinical isolate, Russia, 1973, serogroup I	IMC
51 (9)	Clinical isolate, Russia, 1963, serogroup I	IMC
U-263	Clinical isolate, Russia, 1972, serogroup I	IMC
U-267	Isolated from house rat, Russia, 1971, serogroup I	IMC
U-272	Isolated from house rat, Russia, 1971, serogroup I	IMC
220-73	Clinical isolate, Russia, 1973, serogroup I	IMC
596-73	Clinical isolate, Russia, 1973, serogroup I	IMC
603-73	Clinical isolate, Russia, 1973, serogroup I	IMC
611-73	Clinical isolate, Russia, 1973, serogroup I	IMC
54	Isolated from house mouse, Russia, 1952, serogroup II	IMC
837	Isolated in 1966, serogroup II	HM
854	Isolated in 1966, serogroup II	HM
861	Isolated in 1966, serogroup II	HM
67	Isolated in 1961, serogroup II	HM
55	Isolated in 1971, serogroup II	IMC
445-73	Clinical isolate, Russia, 1973, serogroup III	IMC
162	Isolated from Tamarisk gerbil, Russia, 1957, serogroup III	IMC
145-x	Clinical isolate, Russia, 1971, serogroup III	IMC
445-73	Clinical isolate, Russia, 1973, serogroup III	IMC
677	Isolated from Libyan jird, Russia, 1973, serogroup III	IMC
449-III-D	Clinical isolate, Russia, 1969, serogroup III	IMC
257	Serogroup III	IMC
55	Serogroup III	IMC
286	Serogroup III	IMC
1500	Serogroup III	IMC
53	Isolated from house rat, Russia, 1961, serogroup IV	IMC
665-IV-D	Clinical isolate, Russia, 1965, serogroup IV	IMC
1421	Isolated from house rat, Russia, 1962, serogroup IV	IMC
2255-71	Clinical isolate, Russia, 1971, serogroup IV	IMC
2098-71	Clinical isolate, Russia, 1971, serogroup IV	IMC
U-270	Isolated from mosquito, Russia, 1971, serogroup IV	IMC
U-274	Isolated from house rat, Russia, 1972, serogroup IV	IMC
U-275	Isolated from house rat, Russia, 1972, serogroup IV	IMC
U-276	Isolated from house rat, Russia, 1972, serogroup IV	IMC
810	Received in 1966, serogroup V	HM
48518-V	Clinical isolate, Russia, 1979, serogroup V	IMC
70	Isolated in 1961, serogroup V	HM
56	Isolated from common vole, Russia, 1954, serogroup V	IMC
2126	Serogroup V	IMC
463	Serogroup VI	IMC
1553	Serogroup VI	IMC
VI-J	Serogroup VI	IMC

*CIP, Collection Institut Pasteur, Paris, France; HM, H. Mollaret (Institut Pasteur, Paris, France); IMC, Institute 'Microbe' Collection, Saratov, Russia.

Antigens. To prepare the heat-stable O-antigens of *Y. pseudotuberculosis* serogroups I–VI, standard procedures were used (Knapp & Weber, 1982; Somov *et al.*, 1990). Briefly, broth cultures of each of the six serogroups were heated at 100 °C for 2 h and centrifuged at 2000 ***g*** for 15 min. The pellets were washed twice with saline, resuspended in saline to a final density of $5{\cdot}0 \times 10^8$ bacterial cells ml^{-1} and used for ELISA or SDS-PAGE.

ELISA. Several modifications of ELISA were used. Indirect ELISA was used for initial screening and after cloning, and for studying the ability of mAbs to recognize the *Y. pseudotuberculosis* serogroup-specific epitopes. ELISA plates (96-well) were sensitized with 100 µl of a suspension containing 1×10^9 bacterial cells (ml PBS)$^{-1}$, and incubated overnight at 4 °C. The following bacterial strains were used: reference strains of *Y. pseudotuberculosis* serogroups I–VI, and 50 different *Y. pseudotuberculosis* strains (Table 1) or other closely related Gram-negative bacteria. The wells were washed three times with washing buffer (PBS containing 0·05 % Tween 20). The wells were then saturated with 1 % BSA in PBS for 1 h at 37 °C and washed as above. Then, 100 µl aliquots of cell culture supernates were added and the plates were reincubated for 1 h at 37 °C. After washing, peroxidase-labelled rabbit anti-mouse IgG (Gamaleya Institute, Russia) diluted in diluting buffer (1 % BSA in PBS) was added and the plates were incubated for 1 h at 37 °C. The wells were washed six times. The substrate was 2,2′-azino-bis(3-ethylbenzthiazoline sulfonic acid) (Sigma) at 22·3 mg ml^{-1} in 0·05 mM citrate buffer, pH 4·0, with 0·0003 % H_2O_2 (100 µl). The OD_{405} was measured in a Titertek Multiscan spectrophotometer (Flow Laboratories). An OD_{405} of 0·2 above the background level was considered to be a positive result. RPMI 1640 medium was used as a negative control.

Indirect double-antibody sandwich ELISA was used for studying the substrate specificity of the mAbs, i.e. their ability to react with protein or carbohydrate epitopes and to recognize conformational or linear epitopes of *Y. pseudotuberculosis* antigens. ELISA plates (96-well) were sensitized with rabbit polyclonal absorbed IgG against *Y. pseudotuberculosis* serogroups I–VI (100 µl per well), purified by affinity chromatography as described previously (Feodorova & Devdariani, 2000), at a protein concentration of 10 µg ml^{-1}. Native, heated or proteinase K-treated (PK-treated) microbial cells of *Y. pseudotuberculosis* reference strains of the relevant serogroup (1×10^9 microbial cells ml^{-1}, 100 µl per well) were used as antigens. After incubation for 1 h at 37 °C and washing, culture supernates containing mAbs were added. The remaining procedures were performed as described above.

SDS-PAGE and immunoblotting. Whole-cell lysates of *Y. pseudotuberculosis* serogroups I–VI ($5{\cdot}0 \times 10^8$ microbial cells per lane) and heat-stable O-antigens obtained from *Y. pseudotuberculosis* serogroups I–VI were subjected to SDS-PAGE according to Laemmli (1970), with a 4 % stacking gel and 12·5 % separating gel. For some experiments, the samples of heat-stable O-antigens were incubated with SDS-PAGE sample buffer at 37 °C instead of 100 °C, according to Ogasawara *et al.* (1985). Electrophoresis was done at a constant current of 35 mA in Tris-glycine buffer, pH 8·3, plus 0·1 % SDS buffer, for around 2·5 h. A set of low-molecular mass markers (Sigma) was used. The gels were counter-stained with Coomassie brilliant blue R 250 (Sigma), 0·2 % (w/v) in 25 % ethanol (v/v), 7 % acetic acid (v/v); for some experiments, gels were electrotransferred to nitrocellulose for Western blot analysis (Feodorova & Devdariani, 2002). The membrane was incubated with the mAbs for 12 h at 4 °C. Protein bands were detected with peroxidase-labelled anti-mouse IgG (Sigma).

RESULTS

Characterization of mAbs

After cloning, 19 stable hybridoma cell lines were selected. Two of them ($1C_7$ and $3B_3$) produced mAbs that were positive with the strains of two serogroups in indirect ELISA (Table 2). Fifteen hybridomas produced mAbs which only reacted strongly with the *Y. pseudotuberculosis* strains of the relevant serogroup. No cross-reaction with other pathogenic yersiniae was observed. Thus, these mAbs recognized serogroup-specific, but not strain-specific, epitopes of *Y. pseudotuberculosis* in ELISA. All the mAbs were directed to different serogroup-specific epitopes; this was confirmed by epitope analysis (data not shown). mAbs directed against three different serogroup-specific epitopes of *Y. pseudotuberculosis* serogroups I, III and VI, and against two different epitopes of serogroups II, IV and V, were obtained.

In addition, two hybridomas ($1D_5$ and $1F_3$) secreted mAbs that reacted with all *Y. pseudotuberculosis* strains of every serogroup used, independently of cultivation temperature, and gave no positive reaction with other bacteria. Both mAbs were directed to the same epitope and showed competition in epitope analysis (data not shown). Thus, mAbs directed to a single species-specific epitope of *Y. pseudotuberculosis* were obtained.

In indirect double-antibody sandwich ELISA, all the mAbs (except $3A_2$) reacted with *Y. pseudotuberculosis* whole cells, but not with PK-treated ones. These mAbs recognized protein epitopes. mAb $3A_2$ reacted with both native and PK-treated *Y. pseudotuberculosis* serogroup I, and recognized either a carbohydrate or a protein–carbohydrate epitope.

When tested in indirect double-antibody sandwich ELISA, all the mAbs reacted with both native and heated *Y. pseudotuberculosis* cells of the relevant serogroups. These mAbs were probably directed to linear sequential, but not conformational, epitopes located on heat-stable proteins of *Y. pseudotuberculosis*.

Band patterns revealed by mAbs on immunoblotting

In immunoblotting of each whole-cell lysate of *Y. pseudotuberculosis* serogroups I–VI with all the mAbs (except mAb $3A_2$), a strong positive reaction was only seen with protein bands (Table 3). When the same PK-treated lysate was used, no positive reaction was observed. mAb $3A_2$ gave strong positive immunoblotting with two peptides [molecular masses 41·2 (I_1) and 38·0 (I_2) kDa] in a whole-cell lysate of *Y. pseudotuberculosis* serogroup I, and two carbohydrate components of molecular masses 38·0 and 19·0 kDa in a PK-treated lysate.

Identification of proteins recognized by mAbs in protein profiles of *Y. pseudotuberculosis* serogroups I–VI

Fig. 1 (top) shows the profiles of *Y. pseudotuberculosis*

Table 2. Specific activity of mAbs produced by recloned hybridomas in indirect ELISA, against serogroups of *Y. pseudotuberculosis* and other organisms

None of the mAbs showed a positive reaction with any of the following organisms, grown at either 28 or 37 °C: *Y. pestis* ($n = 8$); *Y. enterocolitica* ($n = 6$); *E. coli* ($n = 2$); *Proteus* spp. ($n = 3$); *Salmonella* spp. ($n = 2$); *Shigella* spp. ($n = 3$).

Serogroup	No. strains	Temp. (°C)	Positive reactions with mAb (%):																	
			$1D_7$	$1F_{10}$	$3A_2$	$3C_3$	$1D_3$	$1C_7$	$5B_9$	$5B_{10}$	$5D_{11}$	$3B_3$	$2B_4$	$3C_2$	$12B_4$	$2B_3$	$1C_4$	$12B_3$	$4C_2$	$1D_5$	$1F_3$
I	15	28	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
	15	37	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
II	6	28	0	0	0	100	0	100	0	0	0	0	0	0	0	0	0	0	0	100	100
	6	37	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	100	100
III	11	28	0	0	0	0	0	0	100	100	100	100	0	0	0	0	0	0	0	100	100
	11	37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
IV	9	28	0	0	0	0	0	100	0	0	0	100	100	100	0	0	0	0	0	100	100
	9	37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
V	5	28	0	0	0	0	0	0	0	0	0	0	0	0	100	100	0	0	0	100	100
	5	37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100
VI	4	28	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100	100	100	100
	4	37	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100

serogroups I–VI grown at 28 °C (lanes 1–6) or at 37 °C (lanes 7–12). Slight cultivation temperature-dependent differences in protein profiles of the same strain were observed.

Proteins I_1 and III_3 were of similar molecular mass, were seen only in lysates of *Y. pseudotuberculosis* serogroups I and III grown at 28 °C (lanes 1 and 3), respectively, and were absent in all remaining strains. Protein I_2 was of a molecular mass similar to those of III_4, IV_2 and V_4, and was observed in all strains independently of cultivation temperature. In lysates of *Y. pseudotuberculosis* serogroups I, III and V, the above-mentioned proteins were seen as major bands, while in lysates of *Y. pseudotuberculosis* serogroups II, IV and VI they appeared as minor bands. Protein I_3 was seen only in *Y. pseudotuberculosis* serogroup I grown at 37 °C (lane 7). Proteins I_4, I_5 and I_6 had molecular masses identical to some proteins seen in lysates of *Y. pseudotuberculosis* serogroups I–III and VI grown at 28 °C; they were absent in lysates of other strains cultured at the same temperature.

The electrophoretic mobility of protein II_1 was similar to that of VI_3, which appeared only in lysates of *Y. pseudotuberculosis* serogroups II and VI independently of cultivation temperature. Protein II_2 was seen in *Y. pseudotuberculosis* serogroup II grown at 28 °C as a major band, while in the same strain grown at 37 °C, it appeared as a minor band (lanes 2 and 7, respectively). Protein II_3 was observed only in *Y. pseudotuberculosis* serogroup II grown at 37 °C. Protein II_4 had a molecular mass similar to that of proteins found in all *Y. pseudotuberculosis* serogroup I–VI strains grown at 28 °C.

Proteins III_1 and III_2 were seen only in *Y. pseudotuberculosis* serogroup III grown at 28 °C. Proteins IV_1 and IV_3 were found only in *Y. pseudotuberculosis* serogroup IV grown at 28 °C. Proteins V_1 and V_2 possessed electrophoretic mobilities similar to those of proteins seen in *Y. pseudotuberculosis* serogroups I–VI independently of cultivation temperature, whereas V_3 appeared only in *Y. pseudotuberculosis* serogroup V grown at 28 °C. Proteins VI_1 and VI_2 were seen only in *Y. pseudotuberculosis* serogroup VI grown at 28 °C.

SDS-PAGE of *Y. pseudotuberculosis* serogroups I–VI O-antigens

Fig. 1 (bottom) shows the protein profiles of heat-stable O-antigens obtained from *Y. pseudotuberculosis* serogroups I–VI (samples were incubated with SDS-PAGE sample buffer at 100 °C). Some of the proteins were denatured during the heating procedure and are observed as a diffuse background. One major protein band was found in *Y. pseudotuberculosis* lysates of each serogroup used, independently of cultivation temperature. This protein had a molecular mass of 38·0 kDa in *Y. pseudotuberculosis* serogroups I, III, IV and V, or 40·0 kDa in *Y. pseudotuberculosis* serogroups II and VI, which was similar to the molecular masses of I_2 (III_4, IV_2 and V_4) and II_1 (VI_3) found in whole-cell lysates of the same *Y. pseudotuberculosis* strains, respectively. In addition, most of the proteins recognized by the mAbs in immunoblotting were also seen by SDS-PAGE.

Table 3. Reaction of mAbs in immunoblotting against *Y. pseudotuberculosis* serogroups I–VI

mAb	Serogroup	Protein bands in kDa (designation)
$1D_7$	I	38·0 (I_2), 26·0 (I_3)
$1F_{10}$	I	58·9 (I_4), 32·7 (I_5), 78·5 (I_6)
$3C_3$	II	40·0 (II_1), 47·3 (II_2)
$1D_3$	II	40·0 (II_1), 29·5 (II_3)
$1C_7$	II, IV	37·7
$5B_9$, $5B_{10}$, $5D_{11}$	III	38·0 (III_4)
$3B_3$	III, IV	40·7 (III_1), 26·0 (III_2); 39·8 (IV_1), 38·0 ± 2 (IV_2)
$2B_4$, $3C_2$	IV	39·8 (IV_1)
$12B_4$	V	77·6 (V_1), 56·9 (V_2), 31·3 (V_3)
$2B_3$	V	38·0 (V_4)
$1C_4$	VI	34·7 (VI_1)
$12B_3$	VI	31·6 (VI_2)
$4C_2$	VI	40·0 (VI_3)
$1D_5$, $1F_3$	I, III–V	38·0
	II, VI	40·0

The same samples were also subjected to SDS-PAGE when they were incubated with SDS-PAGE sample buffer at 37 °C before analysis (Ogasawara *et al.*, 1985). Although the same proteins could be seen in the profiles, their molecular masses were significantly lower than those found in the above-described experiment.

DISCUSSION

In this study, a panel of species- or serogroup-specific mAbs to surface antigens of whole cells of *Y. pseudotuberculosis* was developed. Interestingly, immunochemical studies of the mAbs showed that all of them (except $3A_2$) were directed to protein epitopes. This was revealed by (i) ELISA – all the mAbs reacted with *Y. pseudotuberculosis* whole cells but not with PK-digested ones; and (ii) immunoblotting – mAbs detected the complementary protein bands. This corresponds with the reported presence of several specific proteins in *Y. pseudotuberculosis*; for example, a 45 kDa heat-stable toxin (Nedashkovskaya *et al.*, 1995), a 103 kDa protein encoded by the chromosomal *inv* gene (Kulyashova *et al.*, 1997) and YopA (Aksenov *et al.*, 1995). Moreover, when humoral response in patients with the generalized forms of pseudotuberculosis was studied, the antibody titres to OMPs were significantly higher than to other antigens, including LPS, in all periods of the disease (Burgasova *et al.*, 1996). The same phenomenon was registered in experimental pseudotuberculosis in both mice and guinea pigs, after immunization of the animals with killed whole cells of *Y. pseudotuberculosis* (Drobkov *et al.*, 1996). Specific proteins were also found in *Y. pestis*, which is closely related to *Y. pseudotuberculosis* (Perry & Fetherston, 1997; Feodorova & Devdariani, 2000). Further evidence that *Y. pseudotuberculosis* possessed specific proteins within each serogroup was obtained by examining the protein profiles of the reference strains of the six serogroups used in SDS-PAGE (Fig. 1, top). In all cases, the proteins were observed to react with the relevant mAbs in immunoblotting of the whole-cell lysates of the reference strains of a certain serogroup of *Y. pseudotuberculosis*. Moreover, the polypeptides were the protein components of heat-stable O-antigens, bearing epitopes complementary to serogroup- or species-specific mAbs and providing the serological specificity of *Y. pseudotuberculosis* (Fig. 1, bottom). These data strongly suggest that proteins have a significant role in the serotyping of *Y. pseudotuberculosis*, as for some other Gram-negative bacteria (Zollinger & Mandrell, 1980; Ogasawara *et al.*, 1985; Perry & Fetherston, 1997; Pokrovsky & Posdeev, 1999; Nandi *et al.*, 2000; Feodorova *et al.*, 2001).

It is known that the expression of OMPs in yersiniae is temperature-regulated and associated with the presence of a virulence plasmid (Cornelis *et al.*, 1998). In our study, all *Y. pseudotuberculosis* strains were cultivated in routine conditions in Hottinger agar without any ion supplements, which facilitate secretion of pLcr-encoded proteins (Cornelis *et al.*, 1998). However, the SDS-PAGE profiles of *Y. pseudotuberculosis* strains grown at 28 or 37 °C exhibited some differences (Fig. 1, top). Some proteins, e.g. I_3, II_3, IV_3 and VI_2, were found in the lysates independently of cultivation temperature and were probably temperature-independent proteins, while others were synthesized by *Y. pseudotuberculosis* strains at 28 °C but not at 37 °C (and vice versa) and appear to be temperature-regulated proteins. The changing ability of *Y. pseudotuberculosis* to synthesize some proteins depending on cultivation temperature was confirmed by ELISA. Most of the mAbs obtained gave strongly positive reactions with the strains grown at 28 °C, and showed no reaction with the same strains grown at 37 °C (Table 2). Thus, synthesis of *Y. pseudotuberculosis* serogroup-specific proteins is dependent on cultivation temperature. This phenomenon is not unique for *Y. pseudotuberculosis* and has been observed in other bacteria, for instance, *Neisseria meningitidis* (Zollinger &

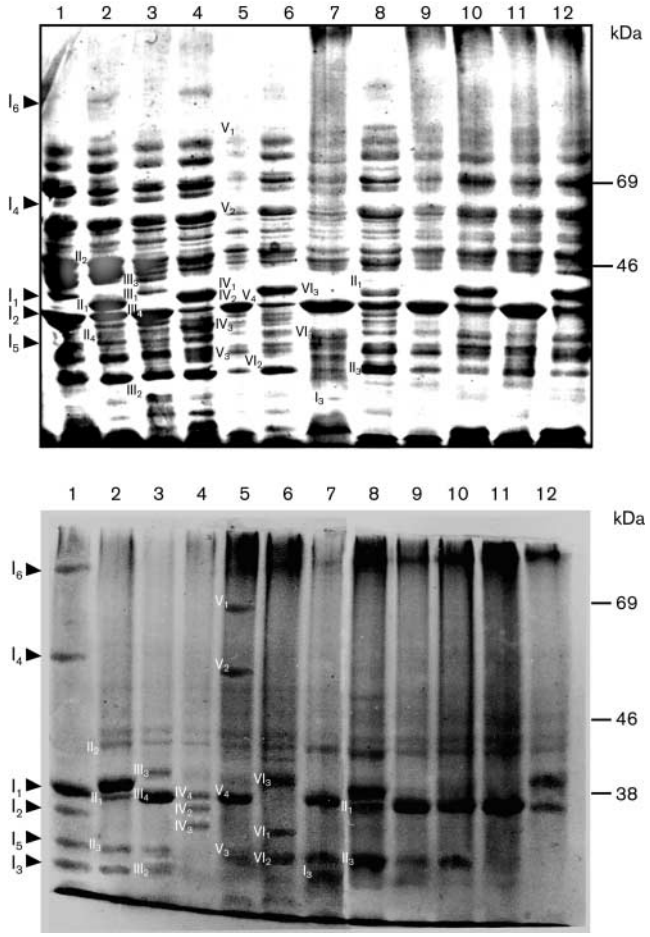

Fig. 1. SDS-PAGE of *Y. pseudotuberculosis* whole-cell lysates (top) and O-antigens (bottom) of serogroups I (lanes 1, 7), II (lanes 2, 8), III (lanes 3, 9), IV (lanes 4, 10), V (lanes 5, 11) and VI (lanes 6, 12), grown at 28 °C (lanes 1–6) or 37 °C (lanes 7–12).

Mandrell, 1977; Beher *et al.*, 1984; Ogasawara *et al.*, 1985). *Y. pestis* is also known to produce species-specific proteins (fraction I, fybrinolysin/coagulase and 'murine' toxin) which can be synthesized at cultivation temperatures of 37, 28/37 or 28/37 °C, respectively (Perry & Fetherston, 1997; Feodorova & Devdariani, 2000). In light of these results, correct serotyping of *Y. pseudotuberculosis* seems to be possible when optimal growth conditions are used.

One significant observation was that *Y. pseudotuberculosis* strains within each serogroup had multiple serogroup-specific epitopes revealed by using the mAbs. This could result from the presence of several different polypeptides with identical serological specificity, or multiple epitopes on the same polypeptide. Indeed, one protein reported previously as a porin (Novikova *et al.*, 1996) or heat-modifiable OMP (Ogasawara *et al.*, 1985) appeared to bear ten different serogroup- or species-specific epitopes. It was identified by alteration of its mobility when electrophoresed by SDS-PAGE without or after heat treatment (Ogasawara *et al.*, 1985). This protein was found in a number of members of the *Enterobacteriaceae*, including all *Yersinia* species, and is responsible for protein type specificity in some bacteria due to the presence of multiple epitopes with different immunoreactivity (Knapp & Weber, 1982; Novikova *et al.*, 1996).

The remaining proteins recognized in immunoblotting by the mAbs were probably monomers of complicated mature protein molecules, bound by disulfide bonds and forming several epitopes with various specificities. This was confirmed by the results of immunoblotting of the mAbs with whole-cell lysates of *Y. pseudotuberculosis* electrophoresed without 2-mercaptoethanol. In all cases, mAbs detected a single complementary protein band (data not shown). All these data led us to conclude that *Y. pseudotuberculosis* is able to synthesize serogroup-specific proteins, consisting of one (in serovars I, III, IV, V and VI), two (in II) or three (in I and V) subunits, stabilized by disulfide bonds in the mature molecule, and in some cases forming several temperature-dependent epitopes with different specificities. Thus, *Y. pseudotuberculosis* can be classified on the basis of OMP antigens. Application of serogroup- and species-specific mAbs may prove useful for the development of highly sensitive, strictly specific and rapid ELISA diagnostic kits for serotyping of *Y. pseudotuberculosis* strains and their differentiation from other microbes, including pathogenic yersiniae.

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