MICROBIAL PATHOGENICITY

Expression of acid-stable proteins and modified lipopolysaccharide of *Yersinia pestis* in acidic growth medium

V. A. FEODOROVA and Z. L. DEVDARIANI

Laboratory of Hybridomas, Russia State Antiplague Research Institute 'Microbe', Saratov, Russia

Growth medium simulating the phagolysosomal environment in which *Yersinia pestis* resides during its intracellular growth *in vivo* was made by acidification of Ca^{2+} -deficient medium. When used for cultivation of *Y. pestis* EV-76 (pLCR⁺; pPst⁺;pFra⁺) and its isogenic derivatives – KM-217 (pLCR⁺;pPst⁻;pFra⁻) and KM-218 (pLCR⁻;Ppst⁻; pFra⁻) – this medium permitted survival and proliferation of viable bacteria without any growth restriction. Moreover, a correlation between the pH of growth medium and bacterial yield was established. Acidification completely inhibited fibrinolytic (pla protease) activity (PAA) of *Y. pestis* carrying pPst and allowed synthesis of specific outer-membrane proteins (Yops) without any degradation by the pla protease. Comparison of whole-cell lysates of the strains tested in PAAG-SDS showed that, in addition to previously described Yops, *Y. pestis* synthesised new acidic proteins which appeared only under acidic conditions and were encoded by pLCR or chromosomally. Some changes in O-specific polysaccharide chains of *Y. pestis* LPS that were dependent on cultivation temperature and pH of the medium were also demonstrated.

Introduction

Yersinia pestis is now considered to be a facultative intracellular parasite of the mammalian reticulo-endothelial system [1–5]. It is capable of survival and growth within macrophages *in vivo* and *in vitro*, residing within phagolysosomes [1–3]. To prevent phagocytosis, bacteria produce a set of specific outer-membrane proteins (Yops) encoded by a 40–50-MDa plasmid (pLCR) common to the three *Yersinia* spp. that are pathogenic for man [4–7]. *In vitro*, Yops are released by pLCR-bearing *Yersinia* strains at 37°C into Ca^{2+} -deficient media in the presence of 20 mM Mg^{2+} and this is associated with growth cessation (i.e., growth restriction) [4, 7–12]. These proteins can be defined easily in both *Y. pseudotuberculosis* and *Y. enterocolitica*. In contrast, it is difficult to detect them in *Y. pestis* as they are rapidly degraded by pla protease (plasminogen activator, fibrinolysin), a product of another *Y. pestis* plasmid (pPst) which is absent from other *Yersinia* spp. [4, 7, 8, 11–13].

This study reports the importance of acidification of the Ca^{2+} -deficient medium routinely used for studying

Yops synthesis by *Y. pestis*. This medium imitates the phagolysosomal environment in which bacteria reside during their intracellular growth *in vivo*. Acidic conditions stimulate bacterial growth and synthesis of Yops and other products by *Y. pestis* and completely inhibit *Y. pestis* plasminogen activator/fibrinolytic activity. Some of the proteins are related by mol. wt to the Yops and YPPs described previously [14] and others may be potential new members of the LCR family or are encoded chromosomally. Many of them are pH-dependent.

Materials and methods

Bacterial strains and bacterial cultivation

The characteristics of *Y. pestis* EV-76 and its isogenic strains are listed in Table 1.

Table 1. *Y. pestis* EV-76 and its isogenic strains used in this study

Strain	Plasmid profile	Reference no.
EV-76	pPst ⁺ ;pLCR ⁺ ;pFra ⁺	13
KM-217	pPst ⁻ ;pLCR ⁺ ;pFra ⁻	13
KM-218	pPst ⁻ ;pLCR ⁻ ;pFra ⁻	13

The strains were grown in Hottinger broth at pH 7.2, 7.0, 6.0, 5.0 and 4.0, containing MgSO₄ 80 mg/L and galactose 0.1% for 24 h at 28°C and then for 24 h at 37°C or for 48 h at 28°C. The strains grown in Hottinger broth, pH 7.2, 7.0, 6.0, 5.0 and 4.0, without any supplements were used as controls.

After cultivation, the bacteria were harvested and washed twice with phosphate-buffered saline (PBS), pH 7.2. Viable cell yields were determined by turbidity measurements and by standard plating techniques. Viable cells were counted and, after killing with sodium merthiolate at a concentration of 1 in 10 000, were used for assays.

Plasminogen activator activity testing

Y. pestis plasminogen activator activity (PAA) was assayed as described by Beesley *et al.* [15].

ELISA

Indirect dot-ELISA was used for studying the ability of all *Y. pestis* strains to synthesise Pla and YPPs encoded by pLCR. Briefly, several drops of bacterial suspension were placed on to nitrocellulose membranes (NCMs), pore size 22 µm (Schleicher-Schuell), and fixed at 110°C for 10 min. After washing three times in PBS and blocking with skimmed milk 3% for 15 min, the NCMs were incubated for 15 min with monoclonal antibodies (MAbs) to Pla [16] or rabbit polyclonal antibodies to YPPs [14], respectively. Peroxidase-labelled anti-mouse or anti-rabbit IgG (Gamaleya Institute, Russia) were employed as conjugates. *O*-Dianisidine (Sigma) was used as a substrate. Finally,

the NCMs were dried and observed for colour differentiation.

SDS-PAGE

Whole-cell lysates of the tested strains grown as described above were subjected to SDS-PAGE according to Laemmli [17] with a stacking 4% gel and separating 12.5% gel. Electrophoresis was done at 35 mA constant current in 25–192 mM Tris-glycine (pH 8.3) plus SDS 0.1% buffer for *c.* 2.5 h. A set of low-mol. wt markers (Sigma) was used. Gels were counterstained with Coomassie Brilliant Blue R 250 (Sigma) 0.2% w/v in ethanol 25% v/v, acetic acid 7% v/v. For some experiments, proteinase K (PK)-treated whole-cell lysates of *Y. pestis* EV-76 grown at 28°C or 37°C were electrophoresed in the same conditions and gels were stained with silver according to Tsai and Frasch [18].

Results

Y. pestis EV-76 plasminogen activator activity

Y. pestis EV-76 grown at 37°C in a medium with neutral or slightly acidic pH expressed PAA, whereas the same strain grown in a medium with lower pH did not have this activity (Table 2). *Y. pestis* EV-76 grown at 28°C did not possess PAA independently of pH.

In dot-ELISA, MAb to Fib gave a strong positive reaction with *Y. pestis* EV-76 grown in media in the pH range 7.2–6.0. No reaction with *Y. pestis* EV-76 grown in media with lower pH was observed (Table 2).

Table 2. Influence of different pH values on proliferative and plasminogen activator activities of *Y. pestis* strains in Hottinger broth

<i>Y. pestis</i> strains	Addition of 20 mM Mg ²⁺ and galactose 0.1%	Medium pH	Cultivation temperature (°C)	Viable cell yield (n × 10 ⁹)	Multiplication ratio*	Plasminogen activator activity	Reaction in dot-ELISA with MAb-Fib
EV-76	–	7.2	28	25.0	1.00	Clot	+
EV-76	+	7.0	28	30.0	1.20	Clot	+
EV-76	+	6.0	28	45.5	1.82	Clot	+
EV-76	+	5.0	28	50.0	2.00	Clot	+
EV-76	+	4.0	28	90.0	3.60	Clot	+
EV-76	–	7.2	37	18.0	1.00	No clot	+
EV-76	+	7.0	37	25.5	1.41	No clot	+
EV-76	+	6.0	37	60.0	3.33	No clot	+
EV-76	+	5.0	37	87.5	4.86	Clot	–
EV-76	+	4.0	37	100.0	5.55	Clot	–
KM-218	–	7.2	37	18.1	1.00	Clot	–
KM-218	+	7.0	37	25.0	1.38	Clot	–
KM-218	+	6.0	37	77.0	4.25	Clot	–
KM-218	+	5.0	37	105.0	5.80	Clot	–
KM-218	+	4.0	37	105.0	5.80	Clot	–
KM-217	–	7.2	37	21.3	1.00	Clot	–
KM-217	+	7.0	37	84.7	3.97	Clot	–
KM-217	+	6.0	37	85.0	3.99	Clot	–
KM-217	+	5.0	37	83.3	3.91	Clot	–
KM-217	+	4.0	37	100.0	4.69	Clot	–

* Multiplication ratio = $\frac{\text{medium pH, indicated on the left, cfu/ml}}{\text{pH 7.2 cfu/ml}}$

Stimulatory effect of acidic pH on growth of *Y. pestis* strains

As shown in Table 2, all *Y. pestis* strains grew well in the media containing 20 mM Mg^{2+} with no calcium at lower pH independently of plasmid composition and cultivation temperature. Moreover, the same phenomenon was found when strain EV-76 was grown in acidified Hottinger broth without any ion supplements. In all cases, bacterial growth was maximal in medium at pH 4.0, and minimal in the same medium at pH 7.0–7.2.

pH and temperature effect on synthesis of pLCR-encoded proteins and other products by *Y. pestis*

Fig. 1 shows whole-cell lysate profiles of the strains grown in the presence of 20 mM Mg^{2+} at 37°C. More proteins were found in all the strains cultivated in media at pH 4.0 than in the same strains cultivated in media at neutral or slightly acidic pH (about 7.0). Some of them (proteins 1–11 with mol. wts 38.0, 28.2, 26.3, 23.9, 22.4, 20.9, 19.5, 16.9, 15.1, 12.6 or 11.5 kDa, respectively) defined in both strain EV-76 and strain KM-217 were absent from strain KM-218. Thus, these proteins were plasmid-encoded (pLCR) polypeptides. Some proteins (nos 1, 2, 4) appeared to be resistant to degradation by pla protease, as they were detected in both strain EV-76 and strain KM-217 independently of pH (Figs. 1 and 2). Protein 3 was seen

in strain EV-76 at every pH tested. In strain KM-217 it was visible only when the strain was grown in medium of pH 4.0–5.0 (Fig. 1, lanes 1 and 2). Protein found in strain EV-76 independently of pH was seen more clearly if this strain was grown in medium at pH 5.0–4.0.

Proteins 5–9 and 11 appeared in strain EV-76 grown only at pH 5.0–4.0 and were absent when this strain was grown in routine conditions (pH 7.2–7.0), being completely degraded by the pla protease [4, 7, 10–14]. Some of these proteins (nos 5–7) were found in strain KM-217 grown in medium at every pH (pH 7.2–4.0). Protein 8 was seen in strain KM-217 grown in medium at pH 4.0. Both proteins 9 and 10 were seen in strain EV-76 at every pH, while protein 10 (similar to protein 11) appeared in strain EV-76 at pH 4.0 only when three times greater concentration of whole-cell lysate of this strain was used (Fig. 1, lane 6). Protein 11 was found in strain KM-217 only at pH 5.0–4.0.

Interestingly, protein 12 with mol. wt 9.1 kDa readily appeared only in strain EV-76 grown at pH 4.0 and was absent from both strain KM-217 and strain KM-218. At the same time, all *Y. pestis* strains produced a protein with mol. wt similar to that of protein 12, but as a minor band. Thus, protein 12 could be a product of pLCR, or any other *Y. pestis* plasmid, or the chromosome. In any case, protein 12 synthesis is pH-dependent.

Fig. 1. SDS-PAGE of whole-cell lysates of *Y. pestis* EV-76 (lanes 6–9), *Y. pestis* KM-217 (1–5) and *Y. pestis* KM-218 (10–12) grown at 37°C in the presence of 20 mM Mg^{2+} , pH 7.2 (lanes 5, 9, 12), 7.0 (4, 8, 11), 6.0 (3), 5.0 (2, 7) or 4.0 (1, 6, 10).

Fig. 2. SDS-PAGE of whole-cell lysates of *Y. pestis* EV-76 (lanes 3–12) or *Y. pestis* KM-218 (1, 2) grown at 28°C (3–7) or at 37°C (1, 2, 8–12), pH 7.2 (3, 8), 7.0 (4, 9), 6.0 (5, 10), 5.0 (1, 6, 11) or 4.0 (2, 7, 12) in the presence of 20 mM Mg^{2+} .

Proteins 13–16 with mol. wts 75.9, 63.1, 48.9 and 36.3 kDa, respectively, were found in *Y. pestis* strains independently of their plasmid composition. All these proteins were seen in strains EV-76, KM-217 and KM-218 grown in medium at neutral pH (7.2, 7.0, 6.0) as minor bands, whereas they appeared in the same strains grown in acidic conditions (pH 5.0–4.0) as major bands.

Proteins 17–21 with mol. wts 27.5, 21.9, 18.2, 15.9 and 12.1 kDa, respectively, were visualised only in strain KM-218 grown at pH 4.0. Thus, these proteins were chromosomally encoded polypeptides synthesised by *Y. pestis* only in acidic conditions. Interestingly, protein 22 with a mol. wt of 83.2 kDa, found in all *Y. pestis* strains as a major band at pH 7.0 and as a minor band at pH 6.0–5.0, disappeared at pH 4.0 in strains EV-76 and KM-218. Proteins 23, 24 and 26 with mol. wts 147.9, 134.9 and 121.0 kDa, respectively, had similar characteristics to protein 22. These proteins also appeared in strain EV-76 grown in medium at a neutral or slightly acidic pH (7.2–6.0) and disappeared in acidic medium (pH 5.0–4.0) (Fig. 2). All these proteins (nos. 23, 24 and 26), as well as proteins 25, 30, 32 and 33 with mol. wts of 125.8, 38.0, 31.6 and 28.2 kDa respectively, were found in strain EV-76 but not in strain KM-218 independently of cultivation temperature. However, when strain EV-76 was grown at 37°C, proteins 23–26 were present in larger amounts (these proteins were visualised as major bands whereas when

strain EV-76 was grown at 28°C they appeared as minor bands). In contrast, protein 25 was seen only in acidic media at pH 5.0–4.0.

Protein 32 appeared in equally large amounts in strain EV-76 independently of cultivation temperature or pH.

Protein 30 was seen only in acidic conditions (pH 5.0–4.0) in strain EV-76 grown at 28°C or 37°C.

Protein 33 was found in strain EV-76 grown at 28°C in medium at pH 6.0.

The remaining proteins (nos. 28, 29, 31, 34–44 with mol. wts of 43.6, 42.0, 37.0, 26.9, 95.5, 87.0, 70.8, 60.3, 57.5, 54.9, 50.1, 44.7, 30.2, 28.2 kDa, respectively) were identified in both strain EV-76 and strain KM-218. Some of them (proteins 28, 34–40, 42) were present in these strains independently of the pH of the medium while proteins 29, 31, 41, 43 and 44 appeared only in acidic conditions. Proteins 28, 31 and 35–44 were seen in strain EV-76 independently of cultivation temperature, whereas protein 29 appeared only when the strain was grown at 37°C. At the same time, many proteins (nos. 28, 35, 37–40, 42 and, probably, no. 34) were degraded by the *pla* protease in strain EV-76 grown at 37°C, as they were clearly visualised in acidic media (pH 4.0–6.0). These proteins were seen as large diffuse zones in media with neutral and slightly acidic pH (pH 6.0–7.2).

LPS profiles of PK-treated *Y. pestis* EV-76 were not completely identical to each other. All possessed the predominant lower mol. wt component corresponding to the core lipid A region and several bands of intermediate mol. wt. However, their number was significantly less in the *Y. pestis* EV-76 grown at pH 4.0 but not at pH 7.2. More changes in O-specific polysaccharide chains were seen in the strains grown without Mg^{2+} than in the same strains grown in the presence of 20 mM Mg^{2+} . In the last case, *Y. pestis* EV-76 grown at pH 4.0–5.0 gave four bands of intermediate mol. wt and it appeared that the bacteria produced at least SR-LPS with short or shortened O-side chains but not R-LPS.

Most of the components found in *Y. pestis* EV-76 appeared in plasmidless *Y. pestis* KM-218. Thus, they were encoded by chromosomal genes. Interestingly, the additional higher mol. wt component was seen in *Y. pestis* EV-76 but not in *Y. pestis* KM-218. Thus, it was probably encoded by a plasmid.

More proteins were also seen in *Y. pestis* EV-76 grown in media without Mg^{2+} , at pH 4.0, than in the same strain cultured at pH 7.2 independently of cultivation temperature (Fig. 3). These were 28.2-, 23.9-, 22.4-, 12.6-, 9.1-, 54.9- and 44.6-kDa proteins with mol. wts similar to proteins 2, 4, 5, 10, 12, 40 and 42, respectively. Proteins 45–57 with mol. wts 83.2, 58.9,

52.5, 40.7, 38.9, 34.7, 25.7, 25.1, 20.9, 17.8, 16.2, 14.5 and 9.3 kDa were also seen.

Discussion

It has been shown previously that expression of V antigen and Yops by yersiniae *in vitro* is maximal at 37°C in media containing 20 mM Mg^{2+} with no Ca^{2+} and is associated with growth restriction [4, 7–12]. These conditions simulate the mammalian intracellular environment in which *Y. pestis* grows in its host target cell – the macrophage [1–4, 8]. When residing within a phagolysosome, bacteria are exposed to acidic hydrolases in acidic conditions (pH 3.5–4.0) [1, 19].

The present study modelled phagolysosomal conditions *in vitro* by acidification of a standard Ca^{2+} -deficient medium and showed that acidic conditions stimulated *Y. pestis* proliferative activity with no growth restriction (Table 2). Therefore, the *Y. pestis* growth restriction *in vitro* described previously [4, 8, 10] is obviously an artifact caused by an artificial growth environment not encountered *in vivo* [4, 10] when bacteria are residing within phagolysosomes [1–3]. The data obtained indicated that *Y. pestis* strains remained viable and proliferated *in vitro* in conditions imitating the phagolysosome environment *in vivo*.

Fig. 3. SDS-PAGE of whole-cell lysates of *Y. pestis* EV-76 grown at 28°C (lanes 1–5) or at 37°C (6–10), pH 7.2 (1, 6), 7.0 (2, 7), 6.0 (3, 8), 5.0 (4, 9) or 4.0 (5, 10) without any supplements.

One fundamental question was whether Yops were really degraded by pla protease in the phagolysosomal environment as reported recently [4, 7, 10–14] or whether Yops were synthesised and functioned in *Y. pestis* containing both pLCR and pPst. The latter supposition was highly probable because: (i) the pLCR permits bacterial survival and growth within phagolysosomes [1–3]; (ii) proteins encoded by this plasmid are released from *Y. pestis* only in the intracellular environment [4–6, 8, 10, 12, 14]; (iii) a significant role for these products in plague pathogenesis is clear [1–5, 7–9, 11, 12, 14]; (iv) as an enzyme, pla protease as well as its precursor easily lost its proteolytic (fibrinolytic) activity at the acidic pH [20, 21]; (v) transformation of the precursor to pla (mature form, fibrinolysin) is possible only at neutral pH but not in acidic conditions [20, 21]. Consequently, *Y. pestis* does not express PAA when residing within the phagolysosome. To confirm this hypothesis, PAA of *Y. pestis* EV-76 grown at 37°C (when both Yops synthesis and PAA are maximal) was studied in media at pH 4.0–7.2. As expected, bacterial growth in acidic conditions produced complete inhibition of *Y. pestis* PAA (Table 2). This observation suggested that, in addition to *pla* (gene), *Y. pestis* possessed a special pH-dependent regulatory mechanism or at least some regulatory gene(s) which blocked its PAA when bacteria resided within phagolysosomes. Moreover, these conditions could allow Yops synthesis and realisation of their functions without degradation by the pla protease. Thus, pla protease could not be responsible for specific degradation of Yops as reported previously [4, 7, 8, 10–14].

To provide additional evidence that no degradation of Yops occurs when bacteria remain in a phagolysosome, whole-cell lysates of *Y. pestis* EV-76 strain bearing all three 'canonical' plasmids (pLCR, pPst, pFra), KM-217 carrying only pLCR and plasmidless strain KM-218 were compared in SDS-PAGE. As shown in Fig. 1, many pLCR-encoded proteins were either absent from strain EV-76 grown at neutral pH (7.2–7.0) or seen as diffuse zones that were probably being degraded by pla protease (Fig. 1, lanes 8, 9). However, the same proteins appeared as distinct clear protein bands in strain KM-217 independently of pH (Fig. 1, lanes 1–5) and in strain EV-76 grown at pH 4.0–5.0 (Fig. 1, lanes 6, 7). Among them some proteins with mol. wts similar to those of Yops and YPPs described previously [4, 5, 8, 11–14] were identified. Confirmation of their identity to each other was determined by dot-ELISA and immunoblotting (data not shown) with rabbit anti-YPPs antibody [14]. Moreover, several potentially new pLCR-encoded proteins (nos. 2, 3, 5–12) were found. Interestingly, the data obtained demonstrated that *Y. pestis* was able to synthesise a few proteins (nos. 7–12) only in acidic pH corresponding to the phagolysosomal environment [19], i.e., pH-dependent acidic proteins. These have not been described previously, but based on mass comparison, proteins 7–12 could be acidic

proteins orfs 85, 60, 61, 54, 74 and 73, respectively, described by Perry *et al.* [22].

Although their functions are unknown at present, these proteins are probably directly involved in blocking of phagocytosis by *Y. pestis*, in preventing the respiratory burst, neutralising acidic hydrolase activity, poisoning macrophages, or promoting escape of bacteria from the phagolysosome into the cell cytoplasm of the macrophage. Nevertheless, these pLCR-encoded products are required for survival of *Y. pestis* within phagolysosomes. The acidic conditions of the phagolysosomal environment make this hypothesis feasible.

The question remained whether the synthesis of Yops (proteins) was regulated by temperature as reported previously [4–12, 13] or not. Comparative analysis of whole-cell lysate profiles of strain EV-76 grown at different pH values at 28°C or 37°C demonstrated that most proteins were expressed in strain EV-76 independently of cultivation temperature (Fig. 2). However, some of them appeared in strain EV-76 grown at 37°C or at lower pH in larger amounts. Moreover, proteins with the same mol. wt (nos. 2, 4, 5, 10, 12, 40 and 42) were found when strain EV-76 was grown only in acidified Hottinger broth without any supplements (Fig. 3). Interestingly, a set of new acidic proteins (nos. 45–57) was also found in *Y. pestis* EV-76 cultured in these conditions independently of temperature of cultivation (Fig. 3). Thus, cultivation temperature and, probably, addition of Mg²⁺ stimulated *Y. pestis* protein synthesis to a lesser extent than acidification of growth media.

A surprising finding in this study was that the acidic conditions promoted synthesis of chromosomally-encoded proteins (Fig. 1) as well as some changes in LPS O-specific polysaccharide chains. Moreover, these chromosomally-encoded proteins were also degraded by pla protease if strain EV-76 was grown in neutral pH (Fig. 1, lane 9) and could easily be visualised in the same strain grown at acidic pH (Fig. 1, lanes 6, 7). This explains why these proteins could not be found previously when pLCR-encoded products were studied [4, 5, 7–14]. Furthermore, there was a perfect correlation between pH of growth medium and differences in O-side chains of *Y. pestis* LPS which become shortened within the phagolysosome. Thus, this antigen could not be considered to be of conserved composition and being an endotoxin it may also be involved in the processes of survival, growth and proliferation of *Y. pestis* in the phagolysosome. Combined with the results presented above, these experiments show the significant role of acidification of Ca²⁺-deficient medium for in-vitro modelling of *Y. pestis* survival during its residence within phagolysosome. These data agree with the observations of Charnetzky and Shuford [1] that the *Y. pestis* life cycles *in vivo* and *in vitro* are not completely analogous in the system used and that the results of earlier studies of *Y. pestis* residing within phagolysosomes in high pH Ca²⁺-deficient medium *in*

vitro should be interpreted with caution. Indeed, the application of acid growth medium provided a closer simulation of the phagolysosomal environment and demonstrated that *Y. pestis* residing within phagolysosomes remains viable, proliferates and produces a set of specific proteins, including some that are pH-dependent.

We thank Dr N. Ye. Teryoshkina for help in preparing the manuscript. We are grateful to Dr M.N. Islyayeva, Dr D. V. Utkin and Mrs Ye. V. Mitina for excellent technical assistance.

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