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Correlation of Pathogenicity and Calcium Dependency of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* with Their Plasmid Content

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Abstract. Derivatives of *Yersinia pseudotuberculosis* and *Y. enterocolitica* were selected by surface plating of the wild type strains on blood agar base containing $MgCl_2$ and sodium oxalate (magnesium oxalate agar). Some of these derivatives lacked the 42 megadalton (Mdal) plasmid and possess the 36 Mdal plasmid, whereas, others lacked both kinds of plasmid. Lack of calcium dependency for growth at 37°C and lack of pathogenicity of the derivatives were demonstrated and indicated that the genetic information for calcium dependency and pathogenicity was carried on the 42 Mdal plasmid of the two species. Whereas, the role (roles) of 36 Mdal plasmid (if any) were not obvious.

Introduction

The genus *Yersinia* which includes three main species *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*, is known to be pathogenic for humans, require calcium for growth at 37°C but not at 26°C, survive in macrophages and possess two surface antigens (V and W) associated with virulence [1, pp. 168–171]. V antigen is a 90–megadalton (Mdal) protein and W antigen is a 140 Mdal liporotein [2].

Y. pestis, the causative agent of bubonic and pneumonic plague, is highly infectious and lethal to humans [1]. Three plasmids (6, 47 and 66 Mdal) were detected in this species [3] and of these, the 47 Mdal contributed to a change in permeability [4] and Ca^{2+} dependence [5]. Whereas, both the 6 and 47 Mdal contributed to virulence [5, 6]. The contribution of the 6 Mdal to virulence, however, was very minor.

Y. pseudotuberculosis can cause enteric disease in humans, characterized by diarrhoea, mesenteric lymphadenopathy and symptoms of appendicitis [7]. *Y. enterocolitica* is well known, also, to cause gastroenteritis which is characterized by

diarrhoea and invasive involvement of the mesenteric lymphatics [8]. The similarity of clinical manifestation and the finding of two common 42 and 36 Mdal plasmids in *Y. enterocolitica* [9] and in *Y. pseudotuberculosis* [10], which are unlike those of *Y. pestis,* suggested that these two species possess a common virulence factor. The aim of the study, therefore, was to determine whether one or both of the two plasmids contributed to the virulence and Ca^{2+} dependence of the two species.

Two wild type and several derivative strains which lacked one or both of the plasmids were used. The plasmid profiles of these strains were correlated with their calcium requirement and virulence.

Materials and Method

Bacterial strains and growth conditions

Yersinia pseudotuberculosis PB1 was obtained from Dr. R.R.Brubaker, Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan. *Y. enterocolitica* 8272 was obtained from Dr. T.F.Wetzler, Department of Environmental Health, University of Washington, Seattle, Washington. Four derivatives were selected from each wild type strain at random by surface plating of the wild type strains on magnesium oxalate agar which consisted of blood agar base (Oxoid), 20 mM $MgCl_2$ and 20 mM sodium oxalate [11]. All inoculated plates were incubated at 37°C for 24 hr. The Ca^{2+} dependent wild type strains were inhibited, whereas, their Ca^{2+} independent derivatives were not. Blood agar base and Higuchi and Carlin [12] broth as modified by Brubaker [13] were used throughout the study. The bacterial strains were maintained at 4°C on blood agar base slants with monthly sub-culture.

Analysis of plasmid content

The plasmid content of the wild type and the derivative strains was analysed by the method of Holmes and Quigley [14]. Bacterial cells were grown overnight in 2 ml of Higuchi and Carlin [12] broth as modified by Brubaker [13], harvested by centrifugation, washed with, and resuspended in, TE buffer. Cells were transferred to a tube containing STET lysis buffer and lysozyme, boiled immediately for 30 seconds, cooled and centrifuged. The plasmid DNA was precipitated by the addition of isopropanol to the resulting supernatant. The sediment was resuspended in TE buffer and one-fourth of the sample was subjected to electrophoresis in a Tris-borate-buffered agarose gel. For details of buffers and method see Holmes and Quigley [14].

Growth on magnesium oxalate agar and blood agar base

An equivalent volume of culture (0.1 ml) containing 150 to 200 colony forming units was harvested from Higuchi and Carlin broth [12] and cultured on 6 plates from each of magnesium oxalate and blood base agars. Three plates, of each medium,

were incubated at 26°C and 3 at 37°C. The average number of colonies was estimated for each medium after an incubation period of 36 hr.

Animal inoculation studies

The wild type strains and their derivatives were grown in Higuchi and Carlin [12] medium as modified by Brubaker [13]. A quantity of 2.5 ml was transferred from each overnight culture to 22.5 ml of a fresh stock of the same medium plus 2.5 mM $CaCl_2$ and were grown for a period of 8 hr at 37°C. The cells were washed and diluted in 0.85% NaCl and 0.1 ml quantities of each dilution were injected intraperitoneally into a total of 5 Swiss Webster mice (0.1 ml/mouse). The LD_{50} of each strain was calculated as described by Reed and Muench [15].

Results

Analysis of plasmid content

Figure 1 shows that 42 and 36 Mdal plasmids were present in the wild type strains of *Y. enterocolitica* and *Y. pseudotuberculosis,* whereas, the derivatives of both species were divided into two types; one type possessed the 36 Mdal plasmid and lacked the 42 Mdal plasmid (lane 3); the other type lacked both plasmids (lane 4). The derivatives PB1S, PB1S2 and PB1S3 of *Y. pseudotuberculosis* PB1 harboured the 36 Mdal plasmid and lacked the 42 Mdal plasmid (Table 1). Moreover, derivative PB1S4 lacked both plasmids. Table 1, also, shows that the mutants 8272S1 and 8272S3 of *Y. enterocolitica* 8272 harbored the 36 Mdal plasmid and lacked the 42 Mdal plasmid, whereas, the mutants 8272S2 and 8272S4 lacked both plasmids.

Growth on magnesium oxalate agar and blood agar base

Table 1 shows that the wild type strains PB1 and 8272 of *Y. pseudotuberculosis* and *Y. enterocolitica* respectively, were not able to grow on magnesium oxalate agar at 37°C, but were able to grow on the same medium at 26°C and on blood agar base at both temperatures. The derivatives of both species, however, were all able to grow on the two media at both temperatures. This indicates that the calcium dependency of the wild type strains was carried on the 42 Mdal plasmid.

Animal inoculation studies

Table 1 shows that the wild type strains PB1 and 8272 of *Y. pseudotuberculosis* and *Y. enterocolitica,* were able to kill 50% of the experimental mice at $\simeq 10^2$ cells/ml, whereas, the derivatives of both species were unable to do so even at a concentration of 10^8 cells/ml. This indicates that the pathogenicity of the wild type strains was carried on the 42 Mdal plasmid.

Table 1. ***Y. pseudotuberculosis*** **and** ***Y. enterocolitica;*** **Their plasmid content, plating efficiency on magnesium oxalate agar and blood agar base and virulence for mice.**

Organism	Strain	Plasmid size (Mdal)	Plating efficiency (average no. of colonies/plate)				LD_{50} (No. of bacteria injected intraperitoneally)
			Magnesium oxalate agar		Blood agar base		
			26°C	37°C	26°C	37°C	
Y. pseudotuberculosis	PB1	36,42	183	2	184	181	1.8×10^2
	PB1S1	36	188	185	189	182	$\geq 10^8$
	PB1S2	36	191	193	186	191	$\geq 10^8$
	PB1S3	36	179	182	184	184	$\geq 10^8$
	PB1S4	None	194	188	190	193	$\geq 10^8$
Y. enterocolitica	8272	36,42	176	1	179	173	1.2×10^2
	8272S1	36	182	180	179	181	$\geq 10^8$
	8272S2	None	181	178	177	179	$\geq 10^8$
	8272S3	36	173	175	175	174	$\geq 10^8$
	8272S4	None	178	176	180	179	$\geq 10^8$

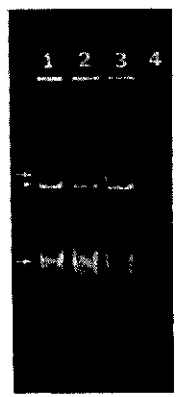

Fig. 1. Plasmid contents of ***Y. pseudotuberculosis*** **PB1 (lane 1),** ***Y. enterocolitica*** **8272 (lane 2) and two of their derivatives (lines 3 and 4). The arrows to the left of lane 1 indicate from top to bottom the position of the 42 Mdal plasmid, 36 Mdal plasmid and chromosomal DNA.**

Discussion

In this study, derivatives of *Y. pseudotuberculosis* PB1 and *Y. enterocolitica* 8272, isolated using magnesium oxalate agar, derivatives lacked the 42 Mdal plasmid while the others lacked both type of plasmids. However, no derivative which possesses only the 42 Mdal plasmid was isolated.

It is well known that the magnesium oxalate binds calcium making it unavailable for the microbial growth [11]. Therefore, the inability of the wild type strains to grow on the magnesium oxalate agar at 37°C and the ability of the derivatives, which lack the 42 Mdal plasmid or lack both plasmids, to grow suggests that the 42 Mdal plasmid is involved in the requirement for Ca^{2+} at 37°C.

This study has shown that the wild type 42 Mdal plasmid bearing strains were virulent for mice, whereas, the derivatives which lack this plasmid were avirulent, so the virulence gene(s) appear to be carried on the 42 Mdal plasmid together with the Ca^{2+} requirement gene(s).

Plasmid-bearing invasive bacteria often produce diverse toxins and to alter their outer membrane [16], so they can avoid the phagocytosis. This 42 Mdal plasmid of *Y. pseudotuberculosis* and *Y. enterocolitica*, accordingly, may similarly enhance the virulence of these two species. However, further studies are required to determine the exact role(s) of this plasmid.

The lack of calcium requirement and virulence by the 36 Mdal plasmid bearing mutants and by the mutants lacking both plasmids show that the 36 Mdal plasmid is cryptic.

The finding of Ca^{2+} requirement and virulence associated with a common plasmid in *Y. pseudotuberculosis* and *Y. enterocolitica* is in agreement with what was reported for *Y. pestis* [5]. The *Y. pestis* plasmid, however, is of different size (47 Mdal).

The virulence and Ca^{2+} dependence are coded for by a particular region of the 47 Mdal plasmid of *Y. pestis* called the Ca^{2+} dependence region [5,6]. The 42 Mdal plasmid may have a similar region. It would be interesting, therefore, to examine the plasmid DNA from both species of Yersiniae with restriction endonucleases.

The virulence plasmid of *Y. pestis* can be introduced into a virulence plasmid free strain of the same species or of *Y. pseudotuberculosis* and that the novel strain is pathogenic and Ca^{2+} dependent [6, 17]. This result is in agreement with the 90% DNA sequence homology of *Y. pestis* and *Y. pseudotuberculosis* [18]. Both species, however, share about 50% DNA sequence homology with *Y. enterocolitica* [19]. Therefore, it would be interesting to study, the mobilization of the virulence and Ca^{2+} dependence plasmid from one species to the other.

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علاقة الإمراضية والاعتماد على الكالسيوم لكلٍ من البكتيريا يرسينيا بسيودوتيوبركيولوسس ويرسينيا انتيروكوليتيكا مع محتواهما البلازميدي

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(أستلم في ١١ صفر ١٤٠٨هـ، قُبل للنشر في ٢٣ شوال ١٤٠٨هـ)

ملخص البحث. لقد تم الحصول على مشتقات لكل من البكتيريا يرسينيا بسيودوتيوبركيولوسس ويرسينيا انتيروكوليتيكا بزراعة السلالات الأبوية على أطباق من بيئة Blood agar base المحتوية على كلوريد المغنيسيوم وأوكسيلات الصوديوم Magnesium oxlate agar . بعض هذه المشتقات لا تملك بلازمد ٤٢ ميجادالتون بينما تملك بلازمد ٣٦ ميجادالتون. أما بقية المشتقات فإنها لا تملك أيا من أنواع البلازمد. لقد وجد أن هذه المشتقات لا تتطلب الكالسيوم للنمو عند ٣٧°م وكذلك وجد أنها غير ممرضة. هذا يبرهن أن الاعتماد على الكالسيوم والإمراضية كليهما منقولين على البلازمد ٤٢ ميجادالتون لكل من النوعين البكتيريين أعلاه، بينما لا يعرف دور البلازمد ٣٦ ميجادالتون.