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NOTES

Pyrazinamidase Activity in *Yersinia enterocolitica* and Related Organisms

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Pyrazinamidase activity was tested in 381 *Yersinia* strains from various ecological and geographical origins and belonging to the following species: *Y. enterocolitica* (five biogroups), *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. aldovae*, *Y. pseudotuberculosis*, and *Y. pestis*. The pyrazinamidase test was negative (Pyz^-) in all bioserogroups of *Y. enterocolitica*, in which is usually harbored the virulence plasmid, and was involved in human or animal diseases. *Y. pseudotuberculosis* and *Y. pestis* were also Pyz^-. The more ubiquitous bioserogroups of *Y. enterocolitica*, without naturally occurring virulence plasmid, and related species were all Pyz^+. Pyrazinamidase activity allowed the separation of the pathogenic North American *Y. enterocolitica* isolates from other nonpathogenic strains within biogroup 1. Similarly, environmental biogroups 3A and 3B were clearly distinguished from pathogenic biogroup 3. However, the pyrazinamidase test was not linked to the presence of the virulence plasmid itself and should not replace the pathogenicity tests to assess the actual virulence of an individual strain. This test proved to be a valuable tool to distinguish potential pathogenic from nonpathogenic strains of *Y. enterocolitica* in epidemiological surveillance programs.

Pyrazine-carboxylamidase (pyrazinamidase) activity has been extensively studied in mycobacteria (12). More recently, it has been proposed as a differential character in *Corynebacterium* species (20). As far as we know, this biochemical property has not been investigated in *Yersinia* species.

Y. enterocolitica includes many serogroups distributed within five biogroups (3). Moreover, four related species have been described recently: *Y. intermedia* (6), *Y. frederiksenii* (22), *Y. kristensenii* (5), and *Y. aldovae* (4). Some strains of *Y. enterocolitica* are involved in human or animal diseases, and their pathogenicity is encoded by a virulence plasmid (26). Strains harboring this plasmid display some growth characteristics like calcium dependency at 37°C (7), autoagglutinability in broth at 37°C (14), and others (10, 11, 13, 16). It has been proposed that such tests distinguish pathogenic from nonpathogenic strains. However, the virulence plasmid can readily be lost in vitro.

Pathogenicity regularly occurs in some bioserogroups, whereas others, mostly of environmental origin, lack the virulence plasmid and may be considered nonpathogenic (10, 17, 24). The purpose of this study was to assess pyrazinamidase activity in *Y. enterocolitica* and related species and correlate it to biochemical profiles, ecological behavior, and potential pathogenicity of the strains.

Bacterial strains. A total of 381 *Yersinia* strains were tested. These strains originated from human, animal, and environmental sources and were isolated in different geographical areas. Species and biogroups were determined by the methods of Brenner et al. (6), Bercovier et al. (3–5), and Ursing et al. (22). Serogrouping was performed according to the O-antigen scheme of Wauters (25). The distribution of species, biogroups, and serogroups of the strains used in this study is given in Table 1.

Biogroup 1 was subdivided into esculin-positive and -negative strains. The latter group included strains of the American type by the method of Shayegani et al. (18, 19). These were mostly of human origin, were all isolated in North America, and belong to known pathogenic serogroups. Other esculin-negative strains were ecologically diversified and originated in different parts of the world. In addition to the strains of biogroup 3, 27 strains belonging to biogroups 3A and 3B, as described by Bercovier et al. (2), were tested.

Three strains of *Y. enterocolitica* belonging to serogroups O:3 (W783), O:9 (W227), and O:5,27 (e24/77) and harboring the virulence plasmid as detected by the method of Kado and Liu (8) were used in parallel with their plasmidless variants.

Y. enterocolitica O:5 biogroup 1 (Y.e.L.35) was tested in parallel with its derivative carrying the virulence plasmid of strain O:9 (W227). The 70-kilobase virulence plasmid of the latter strain was transferred to strain Y.e.L.35 by a transposon-mediated mobilization procedure (Laroche, Bakour and Cornelis, unpublished data).

Calcium dependency at 37°C was determined by the method of Gemski et al. (7). Autoagglutination at 37°C was determined by the method of Laird and Cavanaugh (14) modified by using tryptic soy broth (Difco Laboratories). Hydrolysis of pyrazinamide by pyrazine carboxylamidase results in the production of pyrazinoic acid which turns brownish pink in the presence of ferrous salts.

Pyrazinamidase activity was detected with tryptic soy agar (30 g; Difco)–yeast extract (3 g; Difco)–pyrazinecarboxamide (1 g; Merck)–Tris-maleate (0.2 M, pH 6, 1000 ml) buffer. The medium was dispensed in 5-ml amounts in tubes (160 by 16 mm). After the tubes were autoclaved, they were slanted for cooling. Slants were inoculated with bacteria grown overnight on tryptic soy agar (Difco) and incubated for 48 h at 25 to 30°C. One milliliter of a 1% (wt/vol) freshly prepared ferrous ammonium sulfate (aqueous) solu-

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TABLE 1. Species, biogroups, and serogroups of *Yersinia* strains tested

Species	Biogroup	No. of strains tested	Serogroup
Y. enterocolitica	1 (Esculin$^+$)	100	O:4; O:5; O:6; O:7,8; O:10; O:14; O:16; O:21; O:22; O:25; O:37; O:41; O:46; O:47; O:57, N.T.
	1 (Esculin$^-$; American types)	35	O:4,32; O:8; O:13a,13b; O:18; O:20; O:21
	1 (Esculin$^-$; others)	15	O:16; O:25; O:41,42; N.T.
	2	51	O:9; O:5,27; O:27
	3	22	O:1,2a,3; O:5,27
	4	62	O:3
	5	6	O:2a,2b,3
	3A	12	O:3; O:7; O:7,19; O:19,36; O:47
	3B	15	O:8; O:10; O:16; O:18; N.T.
Y. intermedia		14	O:17; O:21,46; O:35; O:37; O:40; O:48; O:52; O:55
Y. frederiksenii		14	O:3; O:16; O:35; O:38; O:44
Y. kristensenii		14	O:11; O:12; O:16; O:46; O:52
Y. aldovae		2	O:17; O:21
Y. pseudotuberculosis		16	I, II, III, IV, V
Y. pestis		3	

tion was then flooded on the slant. A reading was made after 15 min; a pink color indicated the presence of pyrazinoic acid and was recorded as a pyrazinamidase-positive (Pyz$^+$) reaction. Negative cultures (Pyz$^-$) remained colorless.

Pyrazinamidase activity in the different *Yersinia* species and biogroups is reported in Table 2. *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, and *Y. aldovae* were all Pyz$^+$, although the results varied according to the biogroups in *Y. enterocolitica* and were negative for *Y. pseudotuberculosis* and *Y. pestis*.

Correlation between the pyrazinamidase test, the bioserogroups of *Y. enterocolitica* and their ecological behavior. The esculin-positive strains of biogroup 1, belonging to various serogroups, were uniformly Pyz$^+$. The esculin-negative strains from North America belonging to American serogroups O:8; O:13a,13b; O:18; O:20; O:21; O:4,32 were Pyz$^-$. However, the other esculin-negative strains, mainly from nonhuman sources and isolated in different areas of the world, were Pyz$^+$, e.g., the esculin-positive strains.

Biogroups 2 through 5, whether from human or animal pathogens, were all Pyz$^-$. Biogroups 3A and 3B, as described by Bercovier et al. (2), include environmental strains from water and terrestrial ecosystems and are occasionally found in humans. Unlike the typical biogroup 3, they are Pyz$^+$.

Correlation between pyrazinamidase activity and pathogenicity tests. Pyz$^+$ strains were always negative for calcium dependency and autoagglutination (Table 2). However, all the strains exhibiting positive virulence tests were Pyz$^-$. Several strains which were negative for calcium dependency and autoagglutination were Pyz$^-$ nevertheless. However, these strains belong to known pathogenic bioserogroups (17, 18). Many of them were calcium dependent when first isolated and may have lost their plasmid through subculturing (24). Hence, there was no correlation between the presence of the virulence plasmid and a negative pyrazinamidase test. The lack of correlation between pyrazinamidase activity and the presence of the virulence plasmid is further evidenced by the fact that variants of pathogenic strains of serogroups O:3 and O:9 without plasmids did not acquire pyrazinamidase activity and a nonpathogenic biogroup 1 strain of serogroup O:5 did not become Pyz$^-$ when the virulence plasmid was introduced into the cells (Table 3).

TABLE 2. Pyrazinamidase activity according to species, biogroups, and properties associated with virulence in *Yersinia* strains

Species	Biogroup	No. of strains tested	Percent positive reactions by the following tests[a]:		
			CD	AAG	PYZ
Y. enterocolitica	1 (Esculin$^+$)	100	0	0	100
	1 Esculin$^-$; (American types)	35	40	74	0
	1 (Esculin$^-$; others)	15	0	0	100
	2	51	71	71	0
	3	22	36	36	0
	4	62	87	87	0
	5	6	66	66	0
	3A	12	0	0	100
	3B	15	0	0	100
Y. intermedia		14	0	0	100
Y. frederiksenii		14	0	0	100
Y. kristensenii		14	0	0	100
Y. aldovae		2	0	0	100
Y. pseudotuberculosis		16	50	50	0
Y. pestis		3	100	100	0

[a] Abbreviations: CD, calcium dependency; AAG, autoagglutination; PYZ, pyrazinamidase test.

Although weak pyrazinamidase activity could be detected by other techniques, even in the negative strains, the quantitative difference of this activity, as observed by the proposed method, gave clear results that could be recorded as positive or negative. It has been noted for many years that not all *Y. enterocolitica* strains are associated with human or animal diseases and that several environmental strains occasionally have been isolated from humans without clinical significance (17, 18, 23). However, the most objective evidence of their pathogenicity is supported by the presence of a virulence plasmid (7, 26) and some phenotypic character-

TABLE 3. Pyrazinamidase in *Yersinia* wild strains and their variants with or without virulence plasmid[a]

Strain	Biogroup (serogroup)	Virulence plasmid	CD	AAG	PYZ
W783	4 (O:3)	+	+	+	−
Variant		−	−	−	−
e24/77	2 (O:5,27)	+	+	+	−
Variant		−	−	−	−
W227	2 (O:9)	+	+	+	−
Variant		−	−	−	−
Y.e.L.35	1 (O:5)	−	−	−	+
Variant		+	+	+	+

[a] Abbreviations: CD, calcium dependency; AAG, autoagglutination; PYZ, pyrazinamidase test.

istics, mainly calcium dependency at 37°C and, to some extent, autoagglutinability at the same temperature (7, 14). These properties are only present in the clinically significant bioserogroups, including biogroup 2 (serogroups O:9 and O:5,27), biogroup 3 (O:1,2,3; O:5,27), biogroup 4 (O:3), biogroup 5 (O:2,3), and some esculin-negative strains of biogroup 1, isolated in North American and belonging to several serogroups, e.g. O:8; O:13a,13b; O:18; O:20; O:21; O:4,32 (1, 10, 15, 18, 21).

The virulence plasmid may be lost in vitro in strains obviously associated with disease (24), in which the presence of the plasmid was proven at the time of isolation. Such strains then behave in the pathogenicity tests like avirulent, environmental strains.

There is a close relationship between Pyz^- strains and strains that belong to a pathogenic group of *Yersinia*. The pyrazinamidase test cannot replace any pathogenicity test, since it is only correlated to the ability of the strain to harbor the plasmid and not to the presence of the plasmid itself. Hence, this test could be useful for distinguishing potential pathogenic from nonpathogenic strains in epidemiological surveys. In this respect this test may be able to allow recognition of the pathogenic North American isolates of biogroup 1 on a more reliable base than did esculin hydrolysis (17, 18), since several other esculin-negative strains belonging to biogroup 1 and lacking any pathogenic characteristics are found all over the world. Pyrazinamidase activity might be an important feature for a subdivision of biogroup 1.

Similarly, the environmental strains of biogroups 3A and B, as described by Bercovier et al. (2), are clearly separated from the pathogenic strains of biogroup 3 by means of the pyrazinamidase test. Biogroup 3 and biogroups 3A and 3B have very similar biochemical profiles by conventional tests, whereas their clinical and ecological significance is quite different. The pyrazinamidase test might lead to a revised assessment of these groups.

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