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NOTES

Siderochrome Production by *Yersinia pestis* and Its Relation to Virulence

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P^+ plague strains contained more siderochome-producing organisms than P^-. Siderochrome enhanced the mouse virulence of an $F1^+Vw^+P1^+P^-Pu^+$ strains, inhibited P1 activity, and could be assayed by a paper disk titration method.

In common with other microbes (2, 4, 7), *Yersinia pestis* formed siderochrome(s) (SID, iron-transporting agents) under low-iron conditions. Brain heart infusion broth (BHIB) (Difco) was deferrated to 0.17 to 0.4 μg of Fe/ml by Al_2O_3 adsorption (1), and the pH was adjusted to 7. To prepare deferrated brain heart infusion agar (DBHIA) plates, agar (1%, Oxoid no. 1) was added. The plates were overlayed with 4.0 ml of melted DBHIA containing 3×10^5 test organisms and incubated at 27 C. Only a limited number of early colonies grew after 3 days. These colonies were SID producers because they became surrounded by satellite colonies after prolonged incubation. An $F1^+VW^+P1^+P^-Pu^+$ strain, EV (6), which gave rise to the least SID producers, was suitable for the following SID assay. Sets of four sterilized paper disks were immersed into doubling iron-free saline dilutions and placed on a single lot of EV-inoculated DBHIA. Control disks were immersed in $FeCl_3$ solution. The reciprocal dilution index of the sample, which diffused from at least three of four disks and stimulated EV colonial growths around them after 3 days at 27 C, was determined as SID titer of the sample.

A 4-day EV DBHIB shaking culture produced a higher SID titer than a static culture (16:4). SID was more active at 27 C than at 37 C (8:0). The titer remained unchanged after heating at 60 C for 30 min (8:8) but disappeared after autoclaving (120 C, 20 min). The activity was stable in the pH range from 4.94 to 7.40 overnight at room temperature. The largest zone was obtained around the disk at pH 6.4. SID was nondialyzable against 0.2 M tris(hydroxymethyl)aminomethane-malate buffer, pH 6.4, and could be concentrated 16-fold or more using ammonium sulfate (40% saturation, pH 6.4).

The relationship of SID production to virulence was studied by intravenously examining the mouse toxicity of acetone-killed organisms and the number of SID producers on a single lot of DBHIA using the same culture (27 C, 2-day cultures on Trypticase soy agar [BBL]). Each strain was tested for the known virulence factors of *Y. pestis* (F1 was determined by the direct fluorescent antibody technique with F1-specific rabbit anti-A1122 serum twice absorbed with a F1-less strain [MII40]; VW, P1, and P were determined according to Surgalla et al. [5]; Pu was omitted). P^+ strains gave at least five times more SID producers than P^- strains. Two species possessing motility (*Y. pseudotuberculosis* and *Y. enterocolitica*) grew confluently on the DBHIA surface (Table 1).

A nontoxic dose of SID, injected intraperitoneally in the form of an arachis oil emulsion, enhanced the mouse virulence of EV (Table 2). The EV growth-stimulating effect of SID was indicated by challenging the survivors with a virulent strain (Yreka).

P1-inhibiting activity of SID was revealed (Fig. 1) by placing two sterile paper disks, one containing SID and the other containing $FeCl_3$ solution, 10 mm away from the chloroform-killed growth of $P1^+$ strains (Alexander or A1122) and then overlaying the indicator pseudotuberculosis strain 14I (5).

P^- mutants derived from two virulent strains gave 73 (Alexander derived) or 327 (MP6 derived) SID producers on the same batch of DBHIA plates, whereas EV formed on the average 59 to 80 on each of four plates. Purified P^+ substrains of these two strains formed thousands of SID producers on DBHIA. Selected EV SID producers gave 100% (815/815) P^- progeny

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TABLE 1. *Interrelationships among SID producer ratio, percentage of pigmented colonies, virulence, and toxicity of eight Y. pestis, two Y. pseudotuberculosis, and one Y. enterocolitica strains*

Strain designation[a]	SID producer ratio[b]	Subcutaneous virulence (LD_{50})[c]		Intravenous mouse toxicity (LD_{50}, μg/mouse)[d]
		Mouse	Guinea pig	
Y. pestis				
Yreka $F1^+VW^+P1^+P^+$ (96.2%)	17.0	13	110	35.4
Alexander $F1^+VW^+P1^+P^+$ (55.3%)	15.0	34	<68	26.8
Bryans $F1^+VW^+P1^+P^+$ (69.1%)	18.5	16	>5,200	30.8
MP6 $F1^+VW^+P1^+P^+$ (92.4%)	32.5	21	120	17.7
EV $F1^+VW^+P1^+P^-$ (0%)	1.0	$>10^6$	$>10^6$	21.8
Al122 $F1^+VW^-P1^+P^-$ (0%)	2.8	$>10^6$	$>10^6$	20.4
Tjiwidej $F1^+VW^-P1^+P^-$ (0%)[e]	2.6	$>10^6$	$>10^6$	28.7
MII40 $F1^-VW^-P1^-P^+$ (99.4%)	93.6	$>10^6$	$>10^6$	40.5
Y. pseudotuberculosis				
14I $F1^-VW^-P1^-P^-$ (0%)	>1,000	$>10^6$	$>10^6$	400.0
222/+ $F1^-VW^+P1^-P^-$ (0%)	>1,000	$>10^6$	$>10^6$	400.0
Y. enterocolitica				
03 $F1^-VW^-P1^-P^-$ (0%)	>1,000	ND[f]	ND	ND

[a] Established virulence factors except Pu were assayed according to Surgalla et al. (5). Percentages in parentheses indicate those of pigmented colonies among 71 to 7,771 (mean 1,823) isolated from the respective strain.

[b] The number of early colonies of the respective strain, being formed before day 3 of incubation at 27 C on a DBHIA plate, divided by the number (1 or 2) of EV strain in the simultaneous experiments using the same lot of DBHIA. Two experimental results were combined.

[c] Mean lethal dose (LD_{50}) was calculated from deaths per ten mice or six guinea pigs on day 14 according to the method of Reed and Muench (3a).

[d] Calculated from deaths per 10 injected mice on day 2 after receiving appropriate dilutions of acetone-dried powder saline suspension according to the method of Reed and Muench (3a).

[e] Presumably, an accidental P^- isolate in our laboratory.

[f] ND, Not done.

TABLE 2. *Virulence-enhancing effect of SID on the P^--attenuated strain EV*

Pretreatment	EV dose	Lethality (deaths/10)	Immune survivors (%)[a]
SID with arachis oil[b]	3.8×10^7	10	ND[c]
SID with arachis oil[b]	3.8×10^6	6[d]	100
SID with arachis oil[b]	3.8×10^5	5[d]	100
SID with arachis oil[b]	3.8×10^4	5	100
SID with arachis oil[b]	3.8×10^3	3	57
SID with arachis oil[b]	3.8×10^2	4	17
SID with arachis oil[b]	3.8×10^1	4	33
Saline with arachis oil[e]	3.8×10^7	10	ND
Saline with arachis oil[e]	3.8×10^6	1[d]	100
Saline with arachis oil[e]	3.8×10^5	0[d]	100
SID without arachis oil[f]	3.8×10^6	0[d]	100
SID, intravenous injection	0	0	ND
SID, intraperitoneal injection	0	0	ND

TABLE 2—*Continued*

[a] Percentage of surviving mice 14 days after challenging the initial survivors with 10^5 Yreka organisms (7,000 mean lethal doses).

[b] One-fourth milliliter of SID (titer 16) was emulsified with an equal volume of arachis oil and injected intraperitoneally into 10 mice.

[c] ND, Not done.

[d] Significant difference ($P < 0.05$) according to Fisher exact test (3) between SID-arachis oil group and either the saline-arachis oil group or control (no injection) group.

[e] One-fourth milliliter of saline was emulsified with an equal volume of arachis oil and injected intraperitoneally into 10 mice.

[f] Two-tenths milliliter of SID (titer 16) was used to suspend and inject the EV organisms intraperitoneally into 10 mice.

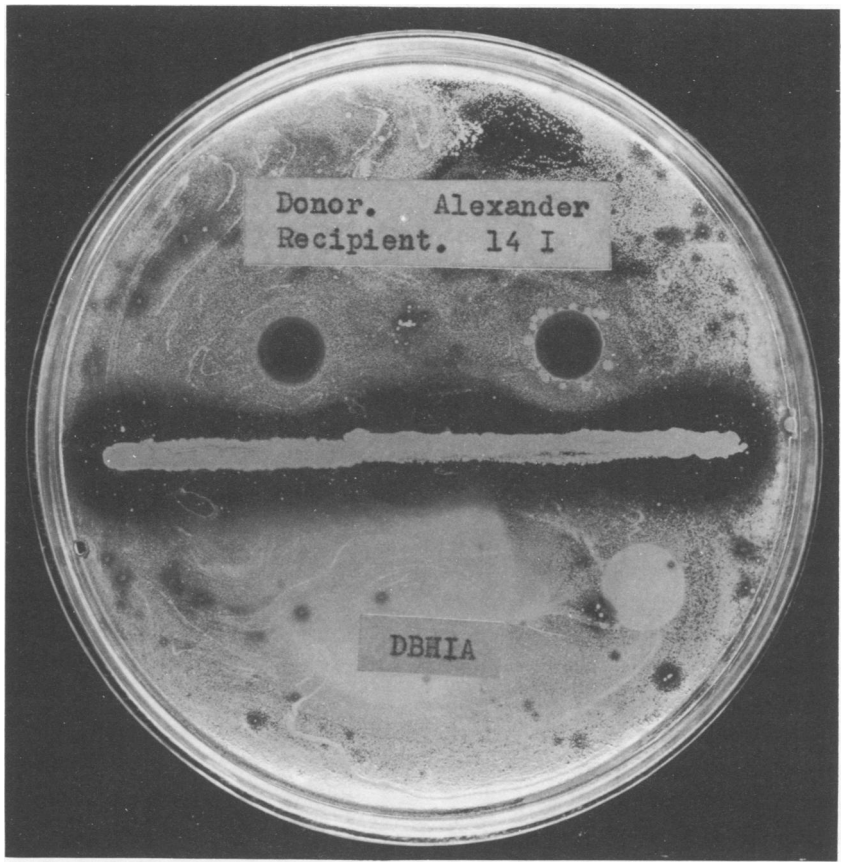

FIG. 1. *P1-inhibiting effect of SID. The growth of the indicator strain 14I around the paper disks, one containing SID sample and the other containing $FeCl_3$ solution, shows that both SID and $FeCl_3$ inhibit P1 activity.*

on Congo red agar. Repeated transfers on DBHIA induced no P^+ phenotype among 406 colonies.

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