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Mutations to Tolerance and Resistance to Pesticin and Colicins in *Escherichia coli* φ†

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The universal colicin-indicator strain *Escherichia coli* φ, unlike *E. coli* strain K-12, is sensitive to pesticin, a bacteriocin produced by wild-type *Yersinia pestis*. Eleven distinct phenotypes of *E. coli* φ mutants were obtained by selection for insensitivity to pesticin, group B colicins, the group A colicin S4, or coliphage T5. Representative isolates from eight of these classes closely resembled resistant receptor mutants (Cir⁻, Fep⁻, and TonA⁻) or tolerant mutants (TonB⁻, ExbB⁻, ExbC⁻, Ivt⁻, and Cmt⁻) described in *Escherichia coli* K-12. The remainder were unique; of these, one resembled TonB⁻ but was also tolerant to colicin S4 (TonB/S4⁻), and the others exhibited specific resistance to either colicin S4 (Sfr⁻) or to pesticin (Psr⁻). All receptor mutants except Psr⁻ remained sensitive to pesticin, whereas TonB/S4, TonB⁻, ExbB⁻, and ExbC⁻ isolates were highly tolerant to this bacteriocin.

As defined by patterns of cross-resistance in mutants of *Escherichia coli* K-12, lethality caused by colicins B, D, G, H, Ia, Ib, M, Q, S1, and V is known to depend upon the presence of envelope proteins, most if not all of which are maximally induced under conditions of iron privation. For example, mutational loss of an outer membrane receptor for the phenolate siderophore enterochelin, a soluble iron carrier produced by *E. coli*, resulted in resistance (inability to absorb) to colicins B and D (*fep*) (12, 22). Similarly, resistance to colicin M and coliphages φ80, T1, and T5 (*tonA*) was associated with the absence of a distinct receptor for exogenous hydroxamate siderophores (23, 37). Colicins Ia, Ib, Q, S1, and V did not absorb to *cir* mutants, which lack a third receptor of unknown physiological function (21, 36) which, like those for binding of siderophores, is induced during growth in iron-deficient medium (13, 35, 37).

As opposed to resistance, tolerance to colicins may occur upon mutational loss of functions required for penetration of absorbed colicins to internal targets or upon modification of such targets. Mutants termed *tonB*, which lack a 40,000-dalton inner membrane polypeptide (32), were tolerant to all of the colicins noted above and unable to propagate coliphages φ80 and T1 (13); this lesion prevents utilization of all known high-affinity systems of iron transport, including those dependent on soluble or cell-bound siderophores or exogenous citrate (19, 24, 29, 38). Accordingly, these colicins are classified within group B (13) as opposed to colicins A, E1, E2, E3, K, L, N, S4, and X of group A (14), where the cognate receptors are not known to be involved in iron metabolism. Other mutants tolerant to group B colicins remained sensitive to coliphages φ80 and T1; these included *exbB* (all colicins of group B), *exbC* (colicins B, D, G, H, and M), *cmt* (colicin M), *ivt* (colicins Ia, Ib, Q, and V), and *cvt* (colicin Q and V) (13, 33, 34). A variety of patterns of resistance and tolerance to group A colicins has been described (14); none of these overlaps those of group B.

Pesticin is a 63,000-dalton polypeptide bacteriocin (25) produced by wild-type *Yersinia pestis* (3); it exhibits nonlytic but lethal *N*-acetylglucosaminidase activity against sensitive bacteria (15). Mutation in *Y. pestis* to non-pesticinogeny (Pst⁻) results in a concomitant loss of distinct invasive enzymes (6), an approximately 6-megadalton plasmid (16), and immunity to the bacteriocin (3). However, the sensitivity of Pst⁻ *Y. pestis* to pesticin was also dependent upon the ability to absorb exogenous low-molecular-weight planar pigmented molecules (Pgm⁺), including hemin (5), a property that is essential for the expression of virulence (27, 28). Other phenotypically Pgm⁻ organisms that are sensitive to pesticin are serotype Ia and Ib strains of *Yersinia pseudotuberculosis* (10), certain serotype 3 and 8 isolates of *Yersinia enterocolitica* (26; unpublished data), and a few strains of *E.*

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coli, including ϕ but not K-12 (7). The purpose of this study was to define pesticin-resistant and -tolerant phenotypes of *E. coli* ϕ, the colicin-indicator strain used extensively by Frederiq (18), to permit comparison with the avirulent pesticin-insensitive Pst^- Pgm^- analog of *Y. pestis*.

MATERIALS AND METHODS

Bacteria. *E. coli* ϕ (20) was used as the indicator organism for all bacteriocins. Colicin-producer organisms were *Citrobacter freundii* strain CA.31 (colicin A), *E. coli* strains AG097 (colicin B), CA.23 (colicin D), CA.42 (colicin E2), CA.38 (colicin E3), CA.46 (colicin G), CA.53 (colicin Ia), K235 (colicin K plus an unknown colicin), CA.18 (colicins B and M), and CA.7 (colicins M and V), *Shigella dispar* strains P14 (colicin E1) and P15 (colicin S4), and *Shigella boydii* strain P1 (colicin S1). *E. coli* strains AG097, CA.23, and CA.38 were obtained from E. Lederberg, D. Pottrell, and D. Beck, respectively; the remainder were received from P. Fredericq. *Y. pestis* strain A1122 was used as the source of pesticin.

Media. Mutants insensitive to colicins and pesticin were selected on nutrient agar (Difco Laboratories, Detroit Mich.) and blood agar base (BBL Microbiology Systems, Cockeysville, Md.), respectively. The latter medium was used to determine the sensitivity of cells to all bacteriocins and coliphages. The ability to excrete enterochelin and the nature of nutritional requirements associated with mutations to bacteriocin insensitivity were assayed with the defined medium (without added iron) of Neidhardt et al. (30), supplemented as previously described (15). Colicins were produced in a medium consisting of 3% type A NZ amine (Sheffield Humko Chemical Co., Lyndhurst, N.J.), 0.025 M K_2HPO_4, 0.01 M citric acid, 0.01 M potassium gluconate, 0.1 mM $FeCl_2$, and 0.01 $MnCl_2$ brought to pH 7 with 10 N NaOH.

Preparation of bacteriocins. Pesticin was purified to homogeneity as previously described (25, 26). Colicin-producing cells were aerated at 37°C on a shaker (400 ml of medium per 2-liter Erlenmeyer flask) until mid-logarithmic growth was achieved. After the addition of mitomycin C (0.4 μg/ml), the organisms were again cultivated for 2 h and then collected by centrifugation at 4°C (11,000 × *g* for 45 min); all subsequent steps were performed in the cold. When antibacterial activity resided primarily in the resulting supernatant fluids (colicins A, B, E1, E2, K, and S1), these were brought to 90% saturation with solid $(NH_4)_2SO_4$, and after equilibration for 30 min, precipitated material was sedimented (14,000 × *g* for 45 min) and dialyzed overnight against 5.0 mM potassium phosphate, pH 7.0 (phosphate buffer). When the majority of activity was cell associated (colicins D, G, Ia, M, S4, and V), the organisms were suspended in 0.05 M Tris-hydrochloride, pH 7.8, and disrupted by treatment for 1 min with an ultrasonic probe (MSE Ltd., London, England) except with colicin M, where disruption was prolonged at 15-s intervals for 20 min as reported by Braun et al. (4). Cellular debris was removed by centrifugation (10,000 × *g* for 10 min), and the resulting extracts were dialyzed overnight against phosphate buffer.

Since *E. coli* K235, CA.7, and CA.18 produce at least two colicins apiece, crude preparations as described above were chromatographed on DEAE cellulose exactly as described previously for purification of pesticin (26). The major antibacterial fraction recovered from strain K235 was termed colicin K. Colicin M appeared in the void volume after chromatography of extracts of *E. coli* strain CA.18 (4) and was similarly detected in strain CA.7 as reported by Fredericq (17). Colicin V was not obtained from the latter until application of NaCl gradient; this preparation may have been contaminated by a second colicin unique to strain CA.7 and reported to possess an antibacterial spectrum identical to that of colicin V (13). Colicin M was further identified by its lysozyme-like action against *E. coli* K-12 (4) and failure to kill *tonA* mutants (37).

Isolation and characterization of mutants. To insure that all mutants arose independently, separate 1 ml cultures were inoculated with about 10 cells of *E. coli* ϕ. When the population reached about 10^7 cells, the cultures were mixed with 3 ml of warm agar and about 400 U (8) of chloroform-sterilized colicin M or 10^7 coliphage; the suspensions were then poured over the surface of sterile nutrient agar plates. Alternatively, similar cultures mixed with warm agar were overlaid directly on lawns of chloroform-killed colicinogenic organisms which had been previously grown for 24 h at 37°C. In both cases, the plates were incubated for 36 to 40 h at 37°C before a single mutant per overlayer was isolated and purified.

The triple agar layer procedure (13) was used to determine whether bacteriocin insensitivity was caused by tolerance or resistance. In this process, bacteriocins diffusing from an initial inoculum of producer organisms are absorbed by tolerant but not resistant cells streaked over a second agar overlayer. Accordingly, indicator cells present in a third overlayer will be killed above resistant but not tolerant organisms. Sensitivity to coliphages was assayed by cross-streaking, and inability to transport enterochelin was determined by the extent of its accumulation 2 h after the organisms entered stationary phase. At this time the cells were removed by centrifugation (10,000 × *g* for 15 min), and 10 ml of the culture supernatant fluid was extracted twice with 10 ml of ethyl acetate. The optical density of these extracts was then determined at 315 nm to obtain an estimate of enterochelin and related degradation products (12). Nutritional deficiencies of mutants were characterized by plating on solid defined medium; this procedure also provided a qualitative estimate of enterochelin excretion, as judged by discoloration of the agar.

RESULTS AND DISCUSSION

Probably the first reports demonstrating a relationship between bacteriocin activity and iron metabolism were those describing the inhibitory effects of Fe^{3+}, hemin, and a hydroxyamate siderophore on the lethality of pesticin (7, 9); apparent pesticin-resistant mutants of *E. coli* ϕ were also shown to have lost sensitivity to coli-

cins B, D, Ia, and S1. In this study we reviewed these discoveries with regard to recent findings concerning Fe^{3+} transport and the lethality of group B colicins. The sensitivity of 362 mutant clones of *E. coli* ϕ to pesticin, 13 colicins, and coliphages ϕ80, T1, and T5 was determined qualitatively, as was their ability to form colonies of normal size on defined medium lacking added iron. Results of this survey were used to classify the mutants by phenotype by criteria previously established for *E. coli* K-12 (13). Of those defined classes, eight were detected in *E. coli* ϕ which, in addition, yielded mutants insensitive to pesticin alone (Psr^-) plus two additional unique phenotypes ($TonB/S4^-$ and Sfr^-) described below. The number of clones of each phenotype determined by these procedures is shown in Table 1.

Since all mutants of a given phenotype, except $TonB/S4^-$, were indistinguishable regardless of the agent used for their selection, only a single type stock of each class was preserved for further study. All mutants studied were as sensitive as the parent to tested colicins of group A except $TonB/S4^-$ and Sfr^- isolates, which were not killed by colicin S4. The sensitivity of the 11 type mutants to coliphages and bacteriocins is defined in Table 2, which also shows the titers of the various colicin preparations used in this study, as determined with *E. coli* ϕ. Added L-tryptophan was necessary for growth of 12% of the $TonB/S4^-$ isolates but this nutrient or other nutrients were not required by other mutants. The probable nature of this requirement for tryptophan is discussed below.

As shown by use of the triple agar layer test, $TonB/S4^-$ and $TonB^-$ isolates were tolerant to pesticin and colicins B, D, G, Ia, M, S1, and V. Both mutants propagated coliphage T5 but not ϕ80 or T1. The only detected differences between them were a slight sensitivity of the $TonB^-$ isolate to massive challenge with colicin B (5,000 U) and a high sensitivity (8 U) to colicin S4. The $TonB/S4^-$ mutant was tolerant to at least 20,000 and 8,000 U of colicin B and S4, respectively. All 68 $TonB/S4^-$ and 23 $TonB^-$ clones formed very small colonies when plated on defined medium lacking added iron; after growth for 2 or 3 days the agar surrounding these colonies acquired a marked orange-purple cast due to the presence of excreted enterochelin.

Also tolerant to pesticin were isolates exhibiting patterns of colicin tolerance similar to those of $ExbB^-$ and $ExbC^-$ mutants of *E. coli* K-12. Distinctions between these phenotypes were partial resistance of $ExbB^-$ cells to coliphages ϕ80 and T1 plus low levels of tolerance to colicins Ia and S1. In contrast, $ExbC^-$ mutants exhibited significantly higher levels of tolerance to colicins B and D. Both mutants formed colonies of nor-

TABLE 1. *Number of isolates of mutant phenotypes of E. coli ϕ recovered by selection with coliphage T5 or various bacteriocins*

Phenotype	No. of isolates recovered with selective agent								
	Bacteriocin								T5
	B plus M	B	D	Ia	M	S1	S4	P[a]	
$TonB/S4^-$	46	0	3	1	12	1	1	4	0
$TonB^-$	9	1	0	0	7	0	0	6	0
$ExbB^-$	0	0	3	2	3	0	0	12	0
$ExbC^-$	0	3	2	0	0	0	0	0	0
Fep^-	0	19	45	0	0	0	0	0	0
Cir^-	0	0	0	18	0	53	0	0	0
Ivt^-	0	0	0	0	0	5	0	0	0
$TonA^-$	0	0	0	0	13	0	0	0	43
Cmt^-	0	0	0	0	9	0	0	0	0
Sfr^-	0	0	0	0	0	0	0	0	0
Psr^-	0	0	0	0	0	0	25	16	0

[a] P, pesticin.

TABLE 2. *Titers of colicins and pesticin tested against type mutants of E. coli ϕ selected for insensitivity to bacteriocins or inability to propagate coliphage T5*

Stock no.	Phenotype	U of bacteriocin required for clearing[a]									Sensitivity[b]		
		B	D	G	Ia	M	S1	S4	V	P[c]	ϕ80	T1	T5
18-30	$TonB/S4^-$	>20,000	>6,000	>4	>4,000	>2,000	>8,000	>8,000	>10	>20,000	0	0	0
36-53	$TonB^-$	5,000	>6,000	>4	>4,000	>2,000	>8,000	8	>10	>20,000	0	0	+
228-14	$ExbB^-$	20	80	>4	5	20	80	1	>10	>20,000	±	±	+
138-1	$ExbC^-$	300	2,000	>4	1	20	1	1	>10	>20,000	+	+	+
139-2	Fep^-	>20,000	>6,000	>4	1	1	1	1	1	1	+	+	+
274-17	Cir^-	1	1	1	>4,000	1	>8,000	1	1	1	+	+	+
303-47	Ivt^-	1	1	1	20	1	40	1	10	1	+	+	+
316-23	$TonA^-$	1	1	1	1	20	1	1	1	1	0	0	0
454-30	Cmt^-	1	1	1	1	20	1	1	1	1	+	+	+
402-25	Sfr^-	1	1	1	1	1	1	>8,000	1	1	+	+	+
11-15	Psr^-	1	1	1	1	1	1	1	1	>20,000	+	+	+

[a] Values are units determined with parent *E. coli* ϕ.
[b] 0, No detectable lysis; ±, partial lysis; +, complete lysis.
[c] P, Pesticin.

mal size on defined medium lacking added iron and did not excrete visibly detectable enterochelin. Mutants identical to Ivt$^-$ and Cmt$^-$ cells of *E. coli* K-12 remained sensitive to pesticin and grew normally on defined medium lacking added iron without evident release of enterochelin.

Psr$^-$ organisms (16 of 38 of the clones selected with pesticin) were resistant to this bacteriocin, as shown by use of the triple agar layer test. These mutants retained sensitivity to all tested coliphages and colicins and formed colonies of normal size on defined medium lacking added iron; release of enterochelin was not detected. Typical TonA$^-$, Cir$^-$, and Fep$^-$ receptor mutants were killed by pesticin. Normal growth of TonA$^-$ and Cir$^-$ isolates on defined medium lacking added iron was noted, whereas excretion of some enterochelin by smaller colonies of Fep$^-$ mutants was generally evident. Although an attempt to select TonB/S4$^-$ mutants with colicin S4 was successful, the majority of isolates recovered by this procedure proved to be resistant rather than tolerant to this colicin, as judged by results of the triple agar layer test. These Sfr$^-$ isolates remained sensitive to all tested coliphages and other bacteriocins used in this study and exhibited no readily apparent lesion in iron metabolism.

Those clones that excreted visible enterochelin always formed small colonies on defined medium. With the exception of the TonB/S4$^-$ mutants that were *trp*, these isolates grew normally without apparent release of enterochelin when supplemented with 10 μM $FeCl_3$. To obtain a direct comparison of the ability to accumulate enterochelin, the excretion of this siderophore in liquid medium was determined. Only cultures of the TonB/S4$^-$, TonB$^-$, and Fep$^-$ isolates contained significant levels of this activity (Table 3).

Accordingly, four distinct mutants were isolated which exhibited patterns of multiple tolerance to pesticin and to colicins known to absorb to different outer membrane receptors involved in the transport of iron. The similarity of three of these mutants to phenotypes described in *E. coli* K-12 (13) prompted their assignment as TonB$^-$, ExbB$^-$, and ExbC$^-$. The fourth phenotype, termed TonB/S4$^-$, exhibited an additional unique tolerance to the group A colicin S4. However, a portion of the TonB/S4$^-$ but not the TonB$^-$ clones were Trp$^-$; *trp* is known to undergo spontaneous deletion with linked *tonB* at high frequency in *E. coli* (11). Only the TonB/S4$^-$ phenotype of *E. coli* ϕ may therefore be entirely analogous to *tonB* mutants as described in *E. coli* K-12. Further study will be required to characterize these pesticin-tolerant mutants fully by genetic mapping and to determine whether *tonB* isolates of *E. coli* K-12 are occasionally tolerant to colicin S4.

TABLE 3. *Doubling times, maximum optical densities, and amount of enterochelin released by wild-type and mutant isolates of E. coli ϕ upon second transfer in liquid defined medium without added iron*[a]

Stock no.	Phenotype	Doubling time (h)	Maximum optical density	U of enterochelin per optical density unit[b]
18–30	TonB/S4$^-$	35	0.125	0.030
36–53	TonB$^-$	35	0.119	0.055
228–14	ExbB$^-$	2	1.185	0.008
138–1	ExbC$^-$	2	1.020	0.015
139–2	Fep$^-$	7	0.269	0.024
274–17	Cir$^-$	2	1.047	0.013
303–47	Ivt$^-$	2	1.191	0.010
316–23	TonA$^-$	2	1.023	0.012
454–30	Cmt$^-$	3	0.780	0.017
402–25	Sfr$^-$	2	1.170	0.014
11–15	Psr$^-$	2	1.017	0.016
	Wild type	2	1.014	0.012

[a] All cultures were supplemented with 0.1 mM L-tryptophan.

[b] Optical density (315 nm) of 1 ml of extracted culture supernatant per optical density unit (620 nm) of that culture.

Typical Ivt$^-$ and Cmt$^-$ mutants of *E. coli* ϕ were obtained but tolerance in these organisms did not extend to pesticin. Similarly, isolates phenotypically indistinguishable from TonA$^-$, Cir$^-$, and Fep$^-$ receptor mutants of *E. coli* K-12 retained sensitivity to pesticin as did hitherto unreported Sfr$^-$ mutants, which evidently lack receptors for colicin S4. Pesticin-resistant Psr$^-$ mutants remained sensitive to all tested coliphages and colicins. This finding indicates that the pesticin receptor was not shared by these antibacterial activities.

Since mutation to *tonB* in *E. coli* ϕ resulted in tolerance to pesticin, the possibility exists that the pesticin receptor, like those of group B colicins, may also be involved in the transport of iron and be repressed by an excess of this cation. This situation would account for the earlier observation that exogenous iron reduced the lethality of pesticin (7). However, the physiological role of the pesticin receptor remains an enigma. It is evidently uncommon in *E. coli* because none of 100 normal hospital isolates examined was sensitive to pesticin (unpublished data). It is probably ubiquitous in *Y. pestis* since sensitivity in this species is dependent upon loss of immunity with retention of the Pgm$^+$ determinant, which is known to be essential for

expression of virulence (28). The mutation to Pgm^- is probably not analogous to that resulting in the $TonB^-$ phenotype of *E. coli* because both Pgm^+ and Pgm^- cells of *Y. pestis* grew in highly iron-deficient medium, transported Fe^{3+} after such growth by a cell-bound process independent of soluble siderophores, and utilized hemin equally well as a sole source of iron (31). Pgm^- mutants of *Y. pestis* may thus resemble Psr^- isolates of *E. coli* ϕ, which lack the pesticin receptor.

Although the ability to accumulate soluble siderophores is clearly essential for the virulence of certain enteric bacteria (35, 40), highly invasive *E. coli* (38, 39) and *Neisseria meningitidis* (1, 2) transport iron via a cell-bound system capable of directly competing with host ligands for bound cation. In addition to its ability to store (27) and utilize hemin as a source of iron (31), *Y. pestis* probably possesses a similar cell-bound mechanism for the transport of Fe^{3+} (31). Further studies are in progress to determine whether these systems are dependent upon a functional *tonB* product and pesticin receptor. An analysis of outer membrane peptides has revealed the presence of at least two components in Pgm^+ cells not present in isogenic Pgm^- derivatives (S. C. Straley and R. R. Brubaker, unpublished data). One of these is probably the pesticin receptor protein.

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