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Nonheritable resistance to chloramphenicol and other antibiotics induced by salicylates and other chemotactic repellents in *Escherichia coli* K-12

(aspirin/acetate/benzoate/multiple drug resistance/chemotaxis)

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ABSTRACT **Phenotypic resistance to chloramphenicol and ampicillin was induced in sensitive *Escherichia coli* K-12 strains during incubation with the following substances: acetate, acetylsalicylate (aspirin), benzoate, dimethyl sulfoxide, 1-methyl-2-pyrrolidinone, and salicylate. In addition, acetylsalicylate and salicylate induced resistance to nalidixic acid and tetracycline. The induction of resistance was highly efficient but varied somewhat with the strain and inducer used. In the presence of inducers, from 10% to 100% of the cells formed colonies on antibiotic media, an increase of 10- to 1000-fold over the controls without inducer. After growth in the absence of these inducers, the cells were normally sensitive to the antibiotics. Thus, the resistance was not due to a heritable change. These inducers also increased the level of chloramphenicol resistance of a strain carrying *cat* (whose gene product inactivates chloramphenicol by acetylation). All of the inducers are chemotactic repellents for *E. coli*, and they are detected by the *tsr* gene product (with the possible exceptions of dimethyl sulfoxide and methylpyrrolidinone, whose modes of detection are not known). Nickel sulfate and cobalt sulfate, repellents that are detected by the *tar* gene product, neither promoted resistance to chloramphenicol nor prevented the induction of resistance by acetylsalicylate. Since several of the inducers are present in common drugs or foods, it may be of medical importance to evaluate their effects on antibiotic therapies.**

Antibiotic resistance in bacteria can be due to genetically determined mechanisms that (*i*) decrease the concentration of the drug in the cell, (*ii*) inactivate the drug, (*iii*) modify the target of the drug, (*iv*) overproduce the target, or (*v*) activate pathways that bypass the need for the target (for reviews, see refs. 1 and 2). In general, the first mechanism leads to a low level of resistance, while the others can lead to high levels of resistance. In some cases, exposure to low concentrations of a particular drug can induce resistance to high concentrations of that drug in cells that are otherwise resistant to only low levels (3, 4).

Previous studies of the effects of various chemicals on transposition indicated that acetate and dimethyl sulfoxide (Me_2SO) increased the frequency of transposants selected on the basis of their drug resistance (5). Recent experiments aimed at understanding this result have tested the effects of these substances by themselves on the resistance of sensitive cells to low concentrations of antibiotics. This report shows that acetate, Me_2SO, acetylsalicylate (AcSal), and several other chemicals induce low level resistance to one or more antibiotics in *Escherichia coli* strain K-12. Unlike the mechanisms discussed above, this resistance is phenotypic (nonheritable). Of particular interest is the fact that all of the inducers are repellents—i.e., they provoke negative chemotaxis in *E. coli*. This suggests that the induction of phenotypic antibiotic resistance may be an adaptive response to the presence of repellents.

MATERIALS AND METHODS

Bacteria. All strains are derivatives of *E. coli* K-12 and were from the National Institutes of Health collection (see Table 1 for references). N117::Tn*9* (N6731) was isolated by infecting strain N117 with λcI857 *bio69 gam210 nin5*::Tn*9* and selecting a transposant on TB/chloramphenicol (Cam) plates as described (5).

Chemicals. Solutions of sodium acetate (Fisher) were neutralized with acetic acid; sodium benzoate (Sigma) and sodium salicylate (Sal) (Fisher) solutions were neutralized with NaOH. Acetylsalicylic acid (Sigma) was dissolved in ethanol at 1 M, and the solution was neutralized with NaOH. $CoSO_4$ (Sigma), $NiSO_4$ (Sigma), indole (Calbiochem), and all other aqueous solutions were sterilized by filtration. Me_2SO (Mallinckrodt), 1-methyl-2-pyrrolidinone (MePyrr) (Aldrich) and methyl salicylate (MeSal) (Sigma) were used as supplied. Cam, ampicillin (Amp), kanamycin sulfate (Kan), and tetracycline (Tet) were from Sigma; nalidixic acid (Nal) was from Calbiochem.

Media. LB medium contained 1% Difco tryptone, 0.5% Difco yeast extract, and 0.5% NaCl (pH 7.4). VBCT medium was Vogel–Bonner citrate medium (6) supplemented with 0.1% Difco tryptone. In these plates (formulated by M. Prival and R. Donnelly of the U.S. Food and Drug Administration), the effective concentration of Cam is higher than in plates lacking citrate (unpublished data). Media were solidified with Difco agar: 1.5% for plates and 0.8% for top agar. Plates were poured immediately after addition of antibiotics and/or test chemicals to melted agar medium cooled to 55°C. In some experiments, the test chemical was added to the top agar containing the bacteria. TMG buffer contained 10 mM Tris, 10 mM $MgSO_4$ and 0.01% gelatin (pH 7.4).

Measurement of Antibiotic Resistance. Fresh overnight cultures grown in LB medium at 31°C were diluted in TMG buffer. Samples containing 10^2 to 10^4 colony-forming units were added to molten top agar (45°C) and immediately poured onto plates containing the indicated concentrations of antibiotics and/or test chemical. The plates were incubated at 31°C or 37°C, and the colonies were counted after 2 or 4 days of incubation. The efficiency of plating (eop) was the number of colonies appearing on test plates divided by the number of colonies appearing on the control plates lacking both antibiotic and test chemical.

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Abbreviations: AcSal, acetylsalicylate; Me_2SO, dimethyl sulfoxide; MePyrr, 1-methyl-2-pyrrolidinone; MeSal, methyl salicylate; Sal, salicylate; eop, efficiency of plating; Amp, ampicillin; Cam, chloramphenicol; Kan, kanamycin; Nal, nalidixic acid; Tet, tetracycline.

Chemotaxis Assays. The "chemical-in-plug" and "test-tube" methods (7) were used to test Me_2SO and MePyrr for activity as repellents. N99 was the tester strain.

RESULTS

Induction of Resistance to Cam by Acetate and Other Repellents. N117 is a Cam-sensitive strain that plated with low efficiency on LB plates containing Cam at 6 μg/ml. The eop increased dramatically, however, when these plates were supplemented with sodium acetate (Fig. 1). At about 25 mM acetate, the eop reached a plateau of about 0.5 or 0.3 for plates incubated at 31°C or 37°C, respectively. In the absence of Cam, the growth of N117 on LB plates was somewhat inhibited by high concentrations of acetate (≥25 mM), as judged by smaller colony size, but no reduction of the eop was found. When cells from a number of the colonies arising on the Cam/acetate plates were grown in LB and then retested, they showed the normal sensitivity of N117 to Cam in the absence of acetate. Thus, the effect of acetate is to induce a nonheritable Cam-resistant phenotype (Cam^R) in a substantial proportion of the N117 population.

Since acetate is known to be a strong chemotactic repellent for *E. coli* (7), a number of other repellents were tested for their ability to induce phenotypic Cam resistance. Table 1 shows that resistance to Cam was induced by several repellents (7) in a variety of strains. The repellents differed in their potency and did not affect all strains equally. AcSal and Sal were highly effective in all the strains tested, inducing resistance in a majority of the cells plated. The fraction of cells rendered resistant to Cam by benzoate varied from 0.1 to 1.0 depending upon the strain used. Interestingly, acetate has generally proved to be a poor inducer of Cam resistance in strains unrelated to N117 (unpublished data). As indicated above, the Cam resistance observed in these experiments was phenotypic because the cells were no longer resistant to Cam when the repellents were not present.

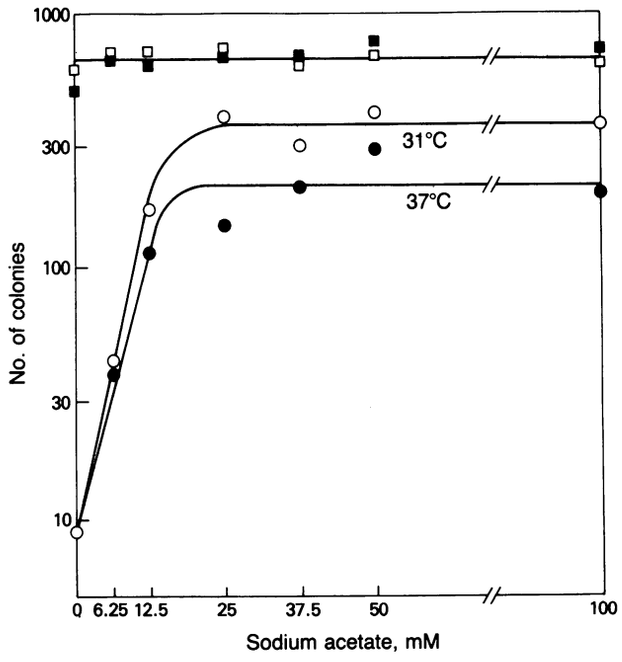

FIG. 1. Induction of Cam resistance by acetate at 31°C (○, □) or 37°C (●, ■). N117, grown overnight in LB medium at 31°C, was diluted and plated on LB (□, ■) or LB/Cam (6 μg/ml) (○, ●) medium containing the indicated amounts of acetate. Colonies were counted after incubation for 60 hr at the indicated temperatures.

Table 1. Effects of inducers on the eop of various strains on LB/Cam (6 μg/ml) plates

		eop with or without inducer			
Strain number*	Ref.	None	AcSal (2.6 mM)	Benzoate (5 mM)	Sal (2.5 mM)
AB1157 (N35)	8	<0.001	0.56	0.11	0.66
AB1157*rec*A13 (N1711)	8	<0.003	0.80	NT	1.02
C600 (N1000)	8	0.004	0.94	0.88	0.91
Hfr H (N156)	8	0.074	0.64	0.48	1.04
28 (N99)	9	<0.002	1.08	1.05	1.05
28*rec*A3 (N100)	9	<0.005	0.85	0.21	1.03
N100(λ) (N117)		<0.003	0.83	0.34	0.72
Y mel C (N720)	8	0.002	1.06	0.68	1.03

Colonies were counted after 4 days incubation at 31°C. NT, not tested.
*Numbers in parentheses are National Institutes of Health strain collection designation.

The effects of varying the concentration of AcSal and Sal on the induction of resistance to Cam at 6 μg/ml in N117 at 31°C and 37°C is shown in Fig. 2 *A* and *B*. In the presence of 0.5 mM AcSal, 10% or 30% of the cells formed Cam-resistant colonies at 37°C or 31°C, respectively. Between 1 and 7.5 mM AcSal, essentially all of the cells were Cam-resistant at either 31°C or 37°C. At 10 mM (31°C) or 20 mM (37°C), <1% of the cells grew on LB medium even in the absence of Cam. Since the AcSal was dissolved in ethanol, the effect of ethanol alone on the induction of Cam resistance was tested. No concentration of ethanol tested (0.5 to 400 μM) increased the ability to form colonies on LB plates with Cam (eop <0.002), although the plating on LB was unaffected.

The concentrations of Sal required to induce Cam resistance were similar to those of AcSal (Fig. 2*B*). However, slightly fewer Cam-resistant colonies were found at 37°C than at 31°C. No reduction of viability due to Sal was found but at 20 mM the colony size was reduced.

The maximum concentrations of Cam at which cells grew depended on the inducer used. Benzoate induced resistance to Cam at 12 μg/ml, and Sal induced resistance to Cam at 25 μg/ml (Table 2). Acetate, Me_2SO, and MePyrr induced resistance to 6 but not to 12 μg of Cam per ml. Both Me_2SO and MePyrr have been found to repel *E. coli* (unpublished data).

Induction of Resistance to Amp, Nal, and Tet. Resistance of N117 to drugs other than Cam was also inducible. Table 3 shows that resistance to Amp was efficiently induced by five of the six repellents tested. The sixth, MeSal, induced a 20-fold increase in plating on LB medium containing Amp despite a toxic effect shown by a 4-fold reduction in plating in the absence of Amp. AcSal and Sal were the strongest inducers of resistance in the strains tested. AcSal and Sal also rendered the cells resistant to Nal at 5 μg/ml and to Tet at 5 (but not 10) μg/ml (Table 3). However, neither acetate nor AcSal nor Sal induced resistance in N117 to as little as 1 μg of Kan per ml. It is clear, nonetheless, that the induction of resistance is not limited to Cam.

Cells that have acquired substantial resistance to Cam, either by mutation or by the acquisition of a Tn*9* (Cam^R) element, can be induced to yet higher levels of resistance with repellents. Strain C6-1, a spontaneous mutant of N117 selected on Cam plates, showed an eop of 0.94 and <0.01 in the presence of Cam at 12 and 25 μg/ml, respectively. In the presence of 5 mM Sal, C6-1 was resistant to Cam at 25 μg/ml (eop = 1.02). Furthermore, strain N6731, an N117::Tn*9* derivative, had an eop of about 0.05 on VBCT plates containing Cam at 20 μg/ml. In the presence of various

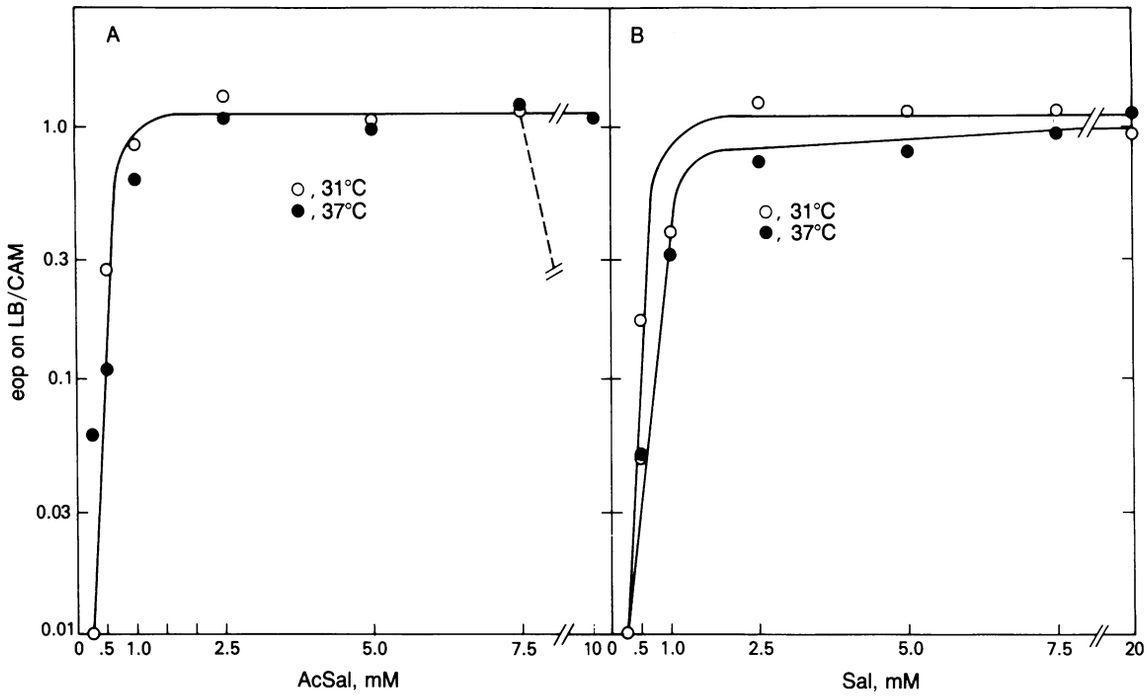

FIG. 2. Induction of Cam resistance by AcSal (*A*) and Sal (*B*). The experiment was as described in Fig. 1. At 0, 0.025, 0.05, and 0.1 mM of either AcSal or Sal, the eop on LB/Cam plates was <0.002.

repellents, its eop approached 1.0 on these plates (Table 4). Note that indole, another repellent of *E. coli* (7), is also an effective inducer.

Sal induced significant resistance to several drugs simultaneously. Less than 10^{-6} of the cells formed colonies in the presence of Tet/Cam, Tet/Nal, or Tet/Cam/Nal (Table 5). However, in the presence of 5 mM Sal, the eop was about 10^6-fold higher. Thus, Sal is an efficient inducer of multiple drug resistance in N117.

Lack of Induction by Co^{2+} and Ni^{2+}. *E. coli* has two known pathways for the detection of repellents. One requires the product of the *tsr* gene and detects acetate, AcSal, benzoate, indole, MeSal, and Sal (11, 12). The other requires the product of the *tar* gene and detects Ni^{2+} and Co^{2+} (11, 12). (Which sensors detect Me_2SO and MePyrr have not yet been determined.) Accordingly, the abilities of $NiSO_4$ and $CoSO_4$ to induce Cam resistance in N117 were tested. Neither repellent was an inducer of Cam resistance (Table 6, lines 1–7). Nevertheless, when present in sublethal amounts, neither prevented AcSal from inducing Cam resistance (Table 6, lines 8–13). AcSal (1 or 2.5 mM) did not prevent the lethality of high concentrations of Co^{2+} (5 mM) or Ni^{2+} (2.5 mM) in the absence of Cam. These results show that the two repellents sensed by the *tar* pathway are not inducers and do not block induction.

Table 2. Effects of inducers on the eop of N117 on LB with different concentrations of Cam

Cam, μg/ml	eop with or without inducer					
	None	Acetate (50 mM)	Benzoate (5 mM)	Me_2SO (140 mM)	MePyrr (2.6 mM)	Sal (5 mM)
0	1.0	1.23	1.05	1.04	1.08	0.82
6	<0.01	1.16*	1.25	0.32*	0.80*	1.05
12	<0.01	<0.01	0.85	<0.01	<0.01	0.84
25	<0.01	<0.01	<0.01	<0.01	<0.01	0.80*

Colonies were counted after 2 days at 31°C.
*The colonies on these plates were minute after 2 days and were not counted until 4 days at 31°C.

DISCUSSION

The antibiotic resistance described here has the following novel features: (*i*) it is not inherited, (*ii*) the compounds that induce it are all chemotactic repellents and are not chemically related to the antibiotics to which resistance is induced, and (*iii*) the structures and modes of action of the antibiotics for which resistance is induced are diverse (1, 2). [Amp inhibits cell-wall synthesis, both Cam and Tet inhibit protein synthesis but act on different ribosomal subunits, and Nal inhibits DNA gyrase activity (13, 14).] Thus, phenotypic antibiotic resistance differs from other resistance mechanisms such as inducible Cam or macrolide/lincosamine/streptogramin resistance, where low levels of an antibiotic induce high levels of resistance to the same or related drugs (3, 4). Since diverse compounds are shown here to induce resistance to diverse antibiotics, it seems unlikely that the inducers directly inactivate the antibiotics, though this possibility is not strictly ruled out. Rather, a more indirect mechanism, such as an influence of inducers on antibiotic entry, seems plausible.

Different chemicals can elicit positive or negative chemotaxis by *E. coli* (for reviews, see refs. 15 and 16). Remarkably,

Table 3. Effects of inducers on the eop of N117 on LB plates supplemented with different antibiotics

Inducer	Conc., mM	eop with or without antibiotic*			
		None	Amp	Nal	Tet
None		1.00	<0.004	<0.004	<0.004
Acetate	50	1.01	0.98	<0.004	<0.004
AcSal	2.5	0.94	1.09	0.46	0.95
Benzoate	5	1.04	0.99	<0.004	<0.004
MePyrr	3.9	1.09	0.76	NT	<0.004
MeSal	9.7	0.24	0.08	NT	<0.004
Sal	5	1.03	0.96	1.04	0.96

Colonies were counted after 4 days at 31°C. NT, not tested; conc., concentration.
*Amp was at 50 μg/ml, whereas Nal and Tet were at 5 μg/ml.

Table 4. Influence of inducers on the eop of N117::Tn*9* (N6731) on VBCT/Cam (20 μg/ml) plates

Inducer	Conc., mM	eop on VBCT/Cam plates
None	—	0.07*
Acetate	25	0.80
Benzoate	5	1.03
Me_2SO_4	140	0.97
Indole	1.7	0.78
Sal	5	0.95

Colonies were counted after 6 days at 31°C. The eop on VBCT, TB, or TB/Cam (20 μg/ml) was about 1.0 ± 0.15 in the presence or absence of inducers.
*These colonies breed true and appear to have tandem duplications of the Tn*9* (unpublished data and ref. 10).

the inducers of drug resistance described here are not only repellents but are repellents that are detected by the *tsr* pathway (11, 12) (though the detectors for Me_2SO and MePyrr are not yet known). Ethanol, $CoSO_4$, and $NiSO_4$, repellents that are not detected by the *tsr* pathway (11, 12), are not inducers. This suggests that *tsr* might mediate the induction of phenotypic antibiotic resistance as an additional adaptive response to repellents. The analysis of appropriate chemotactic mutants should help resolve this question.

Alternatively, the inducers might act on some other cellular component. Acetate, benzoate, and the salicylates are membrane-permeant weak acids. Their effect on cells is to decrease the internal pH, the pH gradient, and the protonmotive force and to increase the membrane potential (17, 18). While their activity as repellents correlates only with their effect on the cell's pH (17, 18), the induction of antibiotic resistance by these weak acids could be due to any of the above mentioned properties. In this regard, it is of interest that dissipation of the membrane potential by valinomycin abolishes the uptake of the aminoglycoside antibiotic gentamycin by *Staphylococcus aureus* (19). Since weak acids increase the membrane potential, this may explain why acetate, AcSal, and Sal did not induce resistance to the aminoglycoside Kan.

The phenomenon of phenotypic antibiotic resistance could be of medical interest. Several of the inducers are frequently ingested (acetate, benzoate, AcSal, and Sal) or applied topically (Me_2SO and MeSal) as foods or analgesics. Furthermore, the concentrations used may approach those found here to induce drug resistance. For example, a blood level of about 2 mM for AcSal or Sal is recommended for treatment of rheumatic fever (20, 21). Thus, studies of the effects of these inducers on antibiotic therapies seem warranted.

These investigations were originally stimulated by the observation that acetate, Me_2SO, and certain detergents increased the frequencies of Kan-, Cam- or Tet-resistant transposants after abortive infection with phage λ variants carrying Tn*5*, Tn*9*, or Tn*10*, respectively (5). Since acetate and Me_2SO are shown here to increase the drug resistance of sensitive cells to Cam, it is reasonable to suppose that these "transposagens" extend the time of bacterial viability in the presence of the drugs and, thereby, increase the time available for transposition to occur. However, this does not explain the effect of acetate on the frequency of Tn*5* transposition, since it did not induce resistance to Kan in sensitive cells.

Table 5. Effects of Sal on the eop of N117 on LB medium with different combinations of antibiotics present

Sal	eop with antibiotics*		
	Cam/Tet	Nal/Tet	Cam/Nal/Tet
None	$<10^{-7}$	7.5×10^{-7}	$<10^{-7}$
5 mM	0.68	0.43	0.18

Colonies were counted after 4 days at 31° C.
*Cam was at 6 μg/ml and Tet and Nal were at 5 μg/ml.

Table 6. Effects of $CoSO_4$ and $NiSO_4$ on the eop of N117 on LB/Cam (6 μg/ml) plates

Addition, mM			Exp. I		Exp. II	
$CoSO_4$	$NiSO_4$	AcSal	LB	LB/Cam	LB	LB/Cam
0	0	0	1.0	<0.002	1.0	<0.004
0.5					0.99	<0.004
1.0			0.77	<0.002	0.80	<0.004
2.5					0.03	<0.004
	0.5				1.05	<0.004
	1.0		0.93	<0.002	0.91	<0.004
	2.5				0.45	<0.004
		1.0	1.11	1.07		
		2.5	1.25	1.06		
1.0		1.0	0.92	0.98		
1.0		2.5	0.85	0.85		
	0.5	1.0			0.98	0.55
	1.0	1.0			0.78	0.64

Colonies were counted after 4 days incubation at 31°C.

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