11-1-2000

Natural Antibiotic Resistance of Bacteria Isolated from Larvae of the Oil Fly, *Helaeomyia petrolei*

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APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2000, p. 4615–4619 Vol. 66, No. 11
0099-2240/00/$04.00+0

Natural Antibiotic Resistance of Bacteria Isolated from Larvae of the Oil Fly, *Helaeomyia petrolei*

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Received 13 March 2000/Accepted 17 August 2000

***Helaeomyia petrolei* (oil fly) larvae inhabit the asphalt seeps of Rancho La Brea in Los Angeles, Calif. The culturable microbial gut contents of larvae collected from the viscous oil were recently examined, and the majority (9 of 14) of the strains were identified as *Providencia* spp. Subsequently, 12 of the bacterial strains isolated were tested for their resistance or sensitivity to 23 commonly used antibiotics. All nine strains classified as *Providencia rettgeri* exhibited dramatic resistance to tetracycline, vancomycin, bacitracin, erythromycin, novobiocin, polymyxin, colistin, and nitrofurantoin. Eight of nine *Providencia* strains showed resistance to spectinomycin, six of nine showed resistance to chloramphenicol, and five of nine showed resistance to neomycin. All 12 isolates were sensitive to nalidixic acid, streptomycin, norfloxacin, aztreonam, cipericillin, pipericillin, and cefotaxime, and all but OF008 (*Morganella morganii*) were sensitive to ampicillin and cefoxitin. The oil fly bacteria were not resistant to multiple antibiotics due to an elevated mutation rate. For each bacterium, the number of resistant mutants per 10^8 cells was determined separately on rifampin, nalidixic acid, and spectinomycin. In each case, the average frequencies of resistant colonies were at least 50-fold lower than those established for known mutator strain ECOR 48. In addition, the oil fly bacteria do not appear to excrete antimicrobial agents. When tested, none of the oil fly bacteria produced detectable zones of inhibition on *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, or *Candida albicans* cultures. Furthermore, the resistance properties of oil fly bacteria extended to organic solvents as well as antibiotics. When pre-exposed to 20 μg of tetracycline per ml, seven of nine oil fly bacteria tolerated overlays of 100% cyclohexane, six of nine tolerated 10% xylene, benzene, or toluene (10:90 in cyclohexane), and three of nine (OF007, OF010, and OF011) tolerated overlays of 50% xylene–50% cyclohexane. The observed correlation between antibiotic resistance and organic solvent tolerance is likely explained by an active efflux pump that is maintained in oil fly bacteria by the constant selective pressure of La Brea's solvent-rich environment. We suggest that the oil fly bacteria and their genes for solvent tolerance may provide a microbial reservoir of antibiotic resistance genes.**

Environments containing high concentrations (10 to 50%, vol/vol) of organic solvents are considered extreme (2). Bacteria able to tolerate such environments have recently been recognized as a subgroup of the extremophiles (2). In this regard, our laboratory has been studying the microbes found in the larval gut of the oil fly, *Helaeomyia petrolei* (9). Thorpe (21) referred to the oil fly as "one of the chief biological curiosities of the world," since its larvae are found exclusively submerged in oil with their larval guts visibly full of petroleum, tar, or asphalt (9, 21). In a previous paper we quantified the microbial gut contents of *H. petrolei* larvae obtained from the asphalt seeps of Rancho La Brea in Los Angeles, Calif. (9). Aerobic incubation of Luria-Bertani (LB), MacConkey, and blood agar plates indicated that each larva contained ca. 2×10^5 heterotrophic bacteria, a value roughly 1,000 times greater than the number of bacteria detected in free oil without *H. petrolei* larvae. All of the bacteria isolated were nonsporeformers and gram negative. Based on representative colony morphologies, 14 isolates were chosen and then identified by using the Enterotube II and API 20E systems, as well as fatty acid analysis (9). Nine of the 14 isolates were identified as *Providencia rettgeri* (9).

Providencia spp. are common agents of nosocomial infections and are now of significant medical interest (5, 8, 20). As a group, they are refractory to treatment with commonly administered antibiotics, disinfectants, and topical heavy metal-containing medications (8). *Providencia stuartii*, which is closely related to and often mistaken for *P. rettgeri* (8), is considered a multiresistant species based on its resistance to a wide range of antibiotics (5, 20). When the mechanisms involved in antibiotic resistance were studied, they were found to be chromosomally encoded (17, 20). In a major survey of antibiotic susceptibility and resistance in *Providencia* spp., Stock and Wiedemann (20) examined 116 clinical isolates of *Providencia* (38 *P. rettgeri* isolates, 35 *P. stuartii* isolates, 23 *Providencia alcalifaciens* isolates, and 20 *Providencia rustigianii* isolates) to determine their susceptibility or resistance to 71 antibiotics (20). They found that *P. stuartii* was the most resistant of the *Providencia* spp., *P. alcalifaciens* and *P. rustigianii* were the most susceptible, and *P. rettgeri* strains were in between (20). For example, all of their clinical isolates of *P. rettgeri* were naturally resistant to tetracyclines and fosfomycin (20).

In the present study we examined 12 bacteria isolated from oil fly larvae (9) to determine their natural susceptibility or resistance to 23 antibiotics. This study was based on the premise that there is a causal overlap between antibiotic resistance and the mechanisms needed for bacterial survival in organic solvents. It is further postulated that because of their relative antiquity—the asphalt seeps at Rancho La Brea have been in existence for at least 40,000 years (18)—the genes for bacterial survival in organic solvents could have provided microbial reservoirs of antibiotic resistance genes in nonpathogenic bacteria. These reservoirs could act as donors (4) of antibiotic resistance to pathogenic bacteria by horizontal transfer (12).

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MATERIALS AND METHODS

Strains and media. Stocks of oil fly isolates OF001, OF003, and OF005 through OF014 (9) were maintained on LB agar supplemented with 2% glucose. Liquid cultures were grown in LB medium plus 2% glucose at 35°C with shaking (200 rpm). The quality control organisms used for antibiotic sensitivity tests, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923, were maintained on blood agar plates. Two mutator analysis controls, ECOR 48 and *E. coli* K-12 strain W3110, were maintained on LB agar.

Antibiotic susceptibility. Antibiotic susceptibility tests were done with BBL Sensi-Disc antimicrobial susceptibility test discs as described in the manufacturer's (Becton Dickinson) product handout revised in December 1997. A complete list of the antibiotics tested is shown in Table 1. LB medium plus 2% glucose was used as the growth medium, and 1.5% agar was added as necessary.

Antimicrobial agent production. Production of an antimicrobial agent by oil fly isolates was tested by preparing lawns of *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, or *Candida albicans* A72 as described above for the susceptibility tests. Oil fly isolates were grown at 30°C in 5 ml of LB medium containing 2% glucose with shaking (200 rpm). After 16.5 h, 1 ml of each culture was centrifuged at room temperature for 2 min at 14,000 rpm in an Eppendorf model 5414 centrifuge. Cell supernatants were decanted and saved, and pellets resuspended in 0.5 ml of 0.85% sodium chloride. Each concentrated cell suspension or supernatant was spotted (20 μl) onto four lawns of potentially susceptible organisms and allowed to soak into the agar. Plates were incubated at 35°C, and zones of inhibition were measured initially after 16 to 18 hours and then again after 48 h.

Mutator percentage analysis. Mutator percentage analysis was performed on the oil fly isolates as described by LeClerc et al. (11). The frequencies with which 10^8 log-phase bacteria became resistant to rifampin (150 μg/ml), spectinomycin (150 μg/ml), and nalidixic acid (20 μg/ml) were measured after 24 h of incubation and compared to the data obtained for the positive and negative controls, (ECOR 48 and *E. coli* K-12 strain W3110, respectively). According to LeClerc et al. (11), a strain is considered to be hypermutable if both the following conditions are met: (i) the frequency of mutation is at least 50-fold greater than that of the negative control (*E. coli* K-12 strain W3110) and (ii) a 50-fold increase in mutation frequency is observed on at least two of three antibiotics, indicating that there is a general mutator, not a mutator specific to just a few genes.

Tetracycline potentiation. The tetracycline potentiation of solvent tolerance was shown by spotting 10^6 cells (15 μl) from an overnight culture onto LB agar plates containing 2% glucose with or without 20 μg of tetracycline per ml. Glass petri dishes were used. The spots were allowed to soak into the agar for 10 to 15 min, and then the plates were overlaid with organic solvent added to a depth of 2 mm. Five different solvents were employed: (i) 100% cyclohexane, (ii) 10% xylene–90% cyclohexane, (iii) 50% xylene–50% cyclohexane, (iv) 10% benzene–90% cyclohexane, and (v) 10% toluene–90% cyclohexane (all by volume). After 6 h the solvents were removed and the plates were incubated at 30°C overnight. The next day the plates were scored as follows: no growth, six or more isolated single colonies, or confluent growth. Three trials were performed with independent cultures on separate days.

RESULTS

Antibiotic resistance. Twelve of the 14 cultures obtained from oil fly larvae (9), including all 9 of those identified as *P. rettgeri*, were tested to determine their disc sensitivity or resistance to 23 commonly used antibiotics (Table 1). All 9 of the *P. rettgeri* strains were highly resistant to tetracycline, vancomycin, bacitracin, erythromycin, novobiocin, polymyxin, rifampin, colistin, and nitrofurantoin, while eight of the nine strains were highly resistant to spectinomycin. In each case, the level of resistance was dramatic; either there were no zones of inhibition around the 6-mm discs or the zones of inhibition were significantly smaller than those defined as indicating resistance in other bacteria (Table 1). The *P. rettgeri* strains also exhibited variable resistance to chloramphenicol and neomycin. The resistance was variable in that only some of the *P. rettgeri* strains were resistant and those that were resistant still showed significant zones of inhibition around the antibiotic-containing discs. In contrast, the *P. rettgeri* strains were highly sensitive to nalidixic acid, streptomycin, norfloxacin, aztreonam, ampicillin, ciprofloxacin, cefoxitin, cefotaxime, and piperacillin and were intermediate or sensitive to kanamycin and tobramycin (Table 1).

Oil fly bacteria as antibiotic producers. Antibiotic-producing microorganisms are often resistant to the antibiotic which

TABLE 1. Sensitivity of the oil fly bacteria to 23 antibiotics[a]

Bacterium or parameter[c]	Sensitivity (diam inhibition zone [mm])[b]																							
	Chl[e]	Kan	Nal	Str	Tet	Nor	Azt	Van	Amp	Cip	Bac	Cef1	Ery	Nov	Pol	Neo	Cef2	Rif	Col	Nit	Tob	Pip	Spe
OF001 (*P. rettgeri*)	R (12)	I (17)	S (22)	S (17)	R (7)	S (27)	S (36)	R (6)	S (23)	S (29)	R (7)	S (26)	R (6)	R (6)	R (6)	R (12)	S (33)	R (10)	R (6)	R (9)	S (16)	S (25)	R (6)
OF003 (*Acinetobacter* sp.)	S (43)	S (23)	S (42)	S (23)	S (35)	S (32)	S (30)	R (8)	S (28)	S (35)	R (6)	S (30)	S (22)	R (11)	S (21)	I (18)	S (37)	S (28)	S (17)	S (27)	S (20)	S (27)	I (17)
OF005 (*P. rettgeri*)	R (10)	I (17)	S (21)	S (18)	R (7)	S (22)	S (32)	R (6)	S (21)	S (25)	R (6)	S (24)	R (6)	R (7)	R (6)	I (13)	S (31)	R (11)	R (6)	R (10)	S (19)	S (26)	R (7)
OF006 (*P. rettgeri*)	S (25)	S (21)	S (23)	S (23)	R (9)	S (25)	S (27)	R (6)	S (24)	S (28)	R (6)	S (26)	R (6)	R (10)	R (7)	I (16)	S (36)	R (12)	R (6)	R (11)	S (17)	S (26)	R (7)
OF007 (*P. rettgeri*)	R (11)	I (17)	S (24)	S (19)	R (6)	S (24)	S (29)	R (6)	S (20)	S (27)	R (6)	S (23)	R (7)	R (8)	R (6)	R (12)	S (29)	R (11)	R (6)	R (9)	S (17)	S (25)	R (6)
OF008 (*M. morganii*)	S (21)	I (19)	S (23)	S (20)	R (10)	S (25)	S (29)	R (6)	R (6)	S (26)	R (6)	R (10)	R (6)	R (18)	R (6)	I (13)	S (26)	R (11)	R (6)	I (16)	S (16)	S (25)	I (17)
OF009 (*P. rettgeri*)	S (22)	I (17)	S (25)	S (17)	R (9)	S (23)	S (29)	R (6)	S (21)	S (25)	R (6)	S (22)	R (6)	I (9)	R (6)	R (12)	S (28)	R (12)	R (6)	R (11)	S (15)	S (25)	I (17)
OF010 (*P. rettgeri*)	R (11)	S (18)	S (26)	S (20)	R (7)	S (27)	S (30)	R (6)	S (20)	S (26)	R (6)	S (24)	R (6)	R (8)	R (6)	I (13)	S (28)	R (12)	R (6)	R (9)	I (13)	S (25)	R (6)
OF011 (*P. rettgeri*)	R (11)	I (17)	S (26)	S (19)	R (7)	S (28)	S (29)	R (6)	S (21)	S (29)	R (6)	S (24)	R (6)	R (8)	R (6)	I (13)	S (29)	R (11)	R (6)	R (10)	I (13)	S (25)	R (6)
OF012 (*P. rettgeri*)	R (11)	I (17)	S (24)	S (19)	R (7)	S (24)	S (26)	R (6)	S (20)	S (26)	R (6)	S (24)	R (6)	R (7)	R (6)	R (11)	S (27)	R (11)	R (6)	R (8)	S (16)	S (25)	R (6)
OF013 (inconclusive)	S (23)	S (19)	S (21)	S (20)	S (22)	S (24)	S (33)	R (6)	S (22)	S (23)	R (6)	S (26)	R (6)	R (13)	R (6)	I (14)	S (29)	R (12)	R (6)	R (12)	S (18)	S (22)	I (16)
OF014 (*P. rettgeri*)	I (13)	I (17)	S (25)	S (18)	R (7)	S (24)	S (31)	R (6)	S (21)	S (26)	R (6)	S (26)	R (6)	R (7)	R (6)	R (11)	S (30)	R (10)	R (6)	R (8)	S (17)	S (24)	R (6)
Resistance[d]	(12)	(13)	(13)	(11)	(14)	(12)	(15)	(14)	(13)	(15)	(8)	(14)	(13)	(17)	(8)	(13)	(14)	(16)	(8)	(14)	(12)	(17)	(14)
Sensitivity	(18)	(18)	(19)	(15)	(19)	(17)	(22)	(17)	(17)	(21)	(13)	(18)	(23)	(22)	(12)	(19)	(23)	(20)	(11)	(17)	(15)	(21)	(18)

[a] Assayed by the BBL Sensi-Disc antimicrobial susceptibility test disc system. All of the procedures used were those recommended by the manufacturer. R, resistant; I, intermediate; S, susceptible. The three control strains specified by the Sensi-Disc instructions, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, and *S. aureus* ATCC 25923, all behaved as expected in the disc assays.

[b] The discs are 6 mm in diameter so an inhibition zone diameter of 6 mm indicates that there was no inhibition at all. All sensitivity assays were conducted in triplicate by using independent cultures. The majority (95%) of the zone means had standard deviations less than 11% of the total diameter. The remaining zone means ± standard deviations still fell within the acceptable range established by Becton Dickinson.

[c] Nine of the 14 oil fly bacteria had been identified (9) as *P. rettgeri*. OF002 to OF004 were *Acinetobacter* strains (9); for convenience, only OF003 was studied here. OF008 was identified as *M. morganii* (9), while the identity of OF013 remained ambiguous (9).

[d] Diameters specified in the Sensi-Disc interpretative chart. For each antibiotic, a culture is defined as resistant if the diameter of the zone of inhibition is less than or equal to the value indicated, susceptible if it is more than or equal to the value indicated, and intermediate if it is in between.

[e] Chl, chloramphenicol (30 μg); Kan, kanamycin (30 μg); Nal, nalidixic acid (30 μg); Str, streptomycin (50 μg); Tet, tetracycline (30 μg); Nor, norfloxacin (10 μg); Azt, aztreonam (30 μg); Van, vancomycin (30 μg); Amp, ampicillin (20 μg); Cip, ciprofloxacin (5 μg); Bac, bacitracin (10 μg); Cef1, cefoxitin (30 μg); Ery, erythromycin (15 μg); Nov, novobiocin (30 μg); Pol, polymyxin B (300 μg); Neo, neomycin (30 μg); Cef2, cefotaxime (30 μg); Rif, rifampin (5 μg); Col, colistin (10 μg); Nit, nitrofurantoin (300 μg); Tob, tobramycin (10 μg); Pip, piperacillin (100 μg); and Spe, spectinomycin (100 μg).

TABLE 2. Mutation frequencies of oil fly bacteria on plates containing rifampin, spectinomycin, and nalidixic acid

Bacterium	Rifampin[a]		Spectinomycin[a]		Nalidixic acid[a]	
	Concn of mutants (CFU/10^8 cells)[b]	Ratio[c]	No. of mutants (CFU/10^8 cells)	Ratio	No. of mutants (CFU/10^8 cells)	Ratio
OF001	25.00 ± 1.63	1.43	Spc[re][e]		1.28 ± 0.35	2.13
OF003	0.87 ± 0.78	0.05	1.06 ± 0.87	6.24	0.14 ± 0.24	0.23
OF005	51.69 ± 31.09	2.96	Spc[r]		33.60 ± 15.08	56.00
OF006	13.60 ± 10.06	0.78	Spc[r]		0.30 ± 0.42	0.50
OF007	16.53 ± 2.28	0.95	Spc[r]		4.06 ± 0.60	6.77
OF008	3.72 ± 0.96	0.21	0.40 ± 0.57	2.35	0.00 ± 0.00	0.00
OF009	13.25 ± 3.37	0.76	0.63 ± 0.88	3.71	1.75 ± 1.96	2.92
OF010	24.44 ± 12.81	1.40	Spc[r]		6.03 ± 2.35	10.05
OF011	4.46 ± 3.28	0.26	Spc[r]		2.39 ± 2.62	3.98
OF012	27.43 ± 9.74	1.57	Spc[r]		3.35 ± 2.45	5.58
OF013	26.03 ± 13.42	1.50	0.26 ± 0.36	1.53	6.98 ± 5.48	11.63
OF014	14.88 ± 7.99	0.85	Spc[r]		2.27 ± 0.23	3.78
ECOR 48[d]	985.08 ± 349.61	56.39	Spc[r]		98.64 ± 43.93	164.40
E. coli K-12 strain W3110[d]	17.47 ± 9.60	1.00	0.17 ± 0.28	1.00	0.60 ± 0.24	1.00

[a] The concentrations of antibiotics used were 150 μg of rifampin per ml, 20 μg of nalidixic acid per ml, and 150 μg of spectinomycin per ml.
[b] The values are the absolute numbers of CFU per 10^8 cells ± standard deviation, based on two to four trials performed with independent cultures. The standard deviations were similar to those recorded by LeClerc et al. (11).
[c] The ratio represents the fold difference calculated for each antibiotic separately relative to the value for the negative control, *E. coli* K-12 strain W3110. The number of CFU per 10^8 cells for each bacterium was divided by the number of CFU per 10^8 cells recorded for the negative control. A mutator strain was defined as an organism which exhibited an increase of approximately 50-fold or greater in the number of mutants per 10^8 cells compared to an established negative control on at least two of three antibiotics (11).
[d] ECOR 48 and *E. coli* K-12 strain W3110 served as the positive and negative controls, respectively. They were employed during each trial and behaved as expected.
[e] Spc[r], spectinomycin resistant.

they produce because they contain a resistance gene as part of the biosynthetic cluster (19). Therefore, one explanation for our finding that the aerobic bacteria obtained from oil fly larvae are predominantly *Providencia* and *Acinetobacter* species (9) is that these bacteria derive a selective advantage by excreting antibiotics that inhibit other microorganisms. Such an occurrence would also explain why our *P. rettgeri* isolates were highly resistant to many commonly used antibiotics (Table 1). However, this scenario did not prove to be correct. When tested on LB agar containing 2% glucose, none of the 12 oil fly bacterial cell suspensions or their supernatants produced detectable zones of inhibition on lawns of *P. aeruginosa*, *E. coli*, *S. aureus*, or the dimorphic fungus *C. albicans* (data not shown).

Oil fly bacteria as mutator strains. Another possible explanation for the prevalence of antibiotic resistance among the *Providencia* strains isolated from oil fly larvae is that they are mutator strains (11). Mutator strains are known to persist in wild-type bacterial populations. For instance, 1 of the 72 strains of *E. coli* which make up the ECOR reference collection (16) is known to be a mutator strain. ECOR 48 was shown (11) to be both MutS$^-$ and hypermutable. By this reasoning, mutator strains would have been selected on the basis of their ability to adapt to the organic solvent stress of the La Brea asphalt seeps and concomitantly the mutator phenotype would have led to the appearance of antibiotic-resistant *Providencia* strains. LeClerc et al. (11) detected MutS$^-$ strains of *E. coli* based on a 50-fold increase in the spontaneous appearance of mutants resistant to rifampin, nalidixic acid, and/or spectinomycin. Accordingly, we analyzed our 12 oil fly bacteria for hypermutability (Table 2) compared to the known positive and negative controls (11), ECOR 48 and *E. coli* K-12 strain W3110, respectively. The experiments measuring resistance to nalidixic acid were straightforward in that all of the oil fly bacteria were initially sensitive to nalidixic acid (Table 1). Although 11 of the oil fly isolates were resistant to the 5-μg rifampin discs (Table 1), MIC analysis determined that the concentration of rifampin used in the mutator analysis (150 μg/ml) was sufficient to inhibit the growth of these organisms (data not shown). The situation with spectinomycin, however, was more complicated. The oil fly isolates that fell in the intermediate category with regard to sensitivity to spectinomycin (Table 1), as well as the spectinomycin-sensitive control organism *E. coli* K-12 strain W3110, were assayed to determine their mutation frequencies. The remaining strains were already resistant to 100 μg of spectinomycin per ml and, therefore, could not be tested.

The mutator status of a bacterium is established by comparing its mutation frequency with that of the designated (11) nonmutator control bacterium *E. coli* K-12 strain W3110. The known hypermutable control strain ECOR 48 showed a 56-fold increase in resistant mutants on rifampin and a 164-fold increase on nalidixic acid (Table 2). In comparison, the oil fly bacteria exhibited increases in mutation frequency of only 0.05- to 2.96-fold on rifampin, 1.53- to 6.24-fold on spectinomycin, and 0.00- to 11.63-fold (with the exception of OF005) on nalidixic acid. Thus, none of the oil fly isolates tested met the requirements of a mutator strain. The only isolate that exhibited an increase in mutator frequency of more than 50-fold was OF005. However, the high mutation rate was restricted to nalidixic acid; on rifampin only a threefold increase was observed (Table 2).

Tetracycline potentiates solvent tolerance. The efflux pumps of gram-negative bacteria expel a remarkably broad range of antimicrobial compounds, including antibiotics, detergents, dyes, and organic solvents (22). The linkage between antibiotic resistance and solvent tolerance in *P. rettgeri* is shown nicely by comparing the abilities of oil fly bacteria to survive in an overlay consisting of 100% solvent with and without prior exposure to tetracycline (Table 3). On LB agar plus 2% glucose, four of the nine isolates tested were able to form colonies after 6 h of exposure to either 100% cyclohexane or a benzene-cyclohexane (1:9) mixture. Three of the isolates (OF007, OF010, and OF011) also survived when they were exposed to xylene-cyclohexane (1:9) and toluene-cyclohexane (1:9) mixtures (Table 3). However, cell survival was dramatically enhanced by prior incubation with 20 μg of tetracycline per ml. With tetra-

TABLE 3. Tolerance of oil fly bacteria to organic solvent overlays with and without prior treatment with tetracycline[a]

Bacterium	No solvent		100% Cyclohexane		10% Xylene–90% cyclohexane		50% Xylene–50% cyclohexane		10% Benzene–90% cyclohexane		10% Toluene–90% cyclohexane	
	Without tetracycline	With tetracycline	Without tetracycline	With tetracycline	Without tetracycline	With tetracycline	Without tetracycline	With tetracycline	Without tetracycline	With tetracycline	Without tetracycline	With tetracycline
OF001	C[b]	C	–	S	–	S	–	–	–	S	–	S
OF005	C	C	–	S	–	–	–	–	–	–	–	–
OF007	C	C	S	C	S	C	–	S	S	C	S	C
OF009	C	C	–	–	–	–	–	–	–	–	–	–
OF010	C	C	S	C	S	C	–	S	S	C	S	C
OF011	C	C	S	C	S	C	–	S	S	C	S	C
OF012	C	C	–	S	–	S	–	–	–	S	–	S
OF013	C	–	S	–	–	–	–	–	S	–	–	–
OF014	C	C	–	S	–	S	–	–	–	S	–	S

[a] A total of 10^6 cells from an overnight culture (15 μl) were spotted onto LB agar plates containing 2% glucose with or without 20 μg of tetracycline per ml. After the spots soaked into the agar (10 to 15 min), the plates were overlaid with solvent added to a depth of 2 mm. Six hours later the solvent was removed, and the plates were incubated at 30°C overnight.

[b] The results are averages of three trials performed with independent cultures on separate days. C, confluent growth; –, no growth; S, six or more single colonies.

cycline present, seven of the nine isolates were able to tolerate 100% cyclohexane, six tolerated benzene-cyclohexane and toluene-cyclohexane (both 1:9), and three tolerated xylene-cyclohexane (1:1). Moreover, the most solvent-tolerant isolates (OF007, OF010, and OF011) exhibited confluent growth after exposure to overlays containing 10% xylene, benzene, or toluene (Table 3).

DISCUSSION

We examined the antibiotic susceptibility and resistance patterns of 12 gram-negative bacteria obtained from oil fly larvae (9) by using 23 commonly used antibiotics. The strains of *P. rettgeri* used were highly resistant to tetracycline, vancomycin, bacitracin, erythromycin, novobiocin, polymyxin, rifampin, colistin, nitrofurantoin, and spectinomycin. With regard to lessons which can be learned from the chemical structures of the antibiotics used in this study, it seems clear that (i) the nine strains of *P. rettgeri* are sensitive to all of the penicillin and cephalosporin derivatives tested; (ii) they are sensitive to antibiotics which target DNA gyrase; (iii) they are resistant to the peptide antibiotics, probably through size exclusion by virtue of being gram negative; and (iv) they are resistant to a diverse collection of comparatively hydrophobic antibiotics (chloramphenicol, erythromycin, nitrofurantoin, novobiocin, rifampin, spectinomycin, and vancomycin), most of which contain aromatic ring systems. In part, this antibiotic resistance pattern is not all that surprising because *Providencia* spp. are known to be highly resistant to antibiotics (5, 8, 20). However, this conclusion was based on examinations of clinical isolates (5, 20), and one has to assume that clinical isolates have been subjected to antibiotic stress. In contrast, we studied bacteria taken from insect larvae, which are natural inhabitants of the La Brea asphalt seeps in California. Oil fly larvae are carnivores whose chief source of food is other insects or animals trapped in the sticky oil (21). Presumably, these bacteria have never been subjected to antibiotic stress.

Oil fly bacteria have, however, been subjected to the energetic stress inherent in a solvent-rich environment (2). For instance, growth of a solvent-tolerant *Pseudomonas putida* strain in 6 mM toluene decreased the maximum cell yield by 33% (6). Part of the energy burden is derived from a membrane-associated organic solvent efflux system (10). The importance of this solvent efflux system is evident from its ability to impart a solvent-resistant phenotype to solvent-sensitive strains of *P. putida* (10). Such efflux systems are involved in extrusion of hydrophobic molecules. Apart from this, they are rather nonspecific in their substrate requirements (14), leading to the expectation that a single efflux system may pump out both organic solvents and antibiotics (22). This expectation is fulfilled in the case of *P. putida* (7); preculturing this bacterium in toluene made it more resistant to hydrophobic antibiotics (7). In addition, we found that the percentage of *P. rettgeri* cells which survived exposure to benzene, xylene, toluene, and cyclohexane was increased by preincubation of the bacteria in the presence of 20 μg of tetracycline per ml.

It is tempting to speculate that the survival of *P. rettgeri* in the La Brea asphalt seeps depends on the ability of the cells to pump out a wide variety of aromatic and polyaromatic hydrocarbons. Although it is not yet known whether organic solvent stress selects for multiple-drug-resistant (MDR) strains in the wild, such selection has been shown to occur in the laboratory, at least for *P. aeruginosa* (13). Work by Li and Poole (13) suggests that an MDR efflux system in *P. aeruginosa* is a common mechanism for achieving organic solvent tolerance and antibiotic resistance. A similar correlation has been reported

for *E. coli* (1), and, based on data presented in this paper, it is likely that the same mechanism is at work in our *P. rettgeri* oil fly isolates. This rationale is strengthened when one considers that the enrichment process at the La Brea asphalt seeps has maintained a constant selective pressure for organisms adapted to organic solvents for over 40,000 years (18).

The evolutionary origins of the genes for antibiotic resistance are not yet known. Bacteria isolated more than 65 years ago, in the preantibiotic age, contained plasmids but lacked the antibiotic resistance genes that we find on those plasmids today (15). Therefore, according to O'Brien et al. (15), the dozens of resistance genes that we now find in various combinations on plasmids of clinical isolates must have either (i) not evolved yet or (ii) been "lurking in more obscure strains," waiting to "emerge from obscure genomes to become deployed on plasmids under the selective pressure of antibacterial usage." We suggest that the oil fly bacteria and their genes for solvent tolerance may be candidates for some of the obscure genes from obscure strains from which current antibiotic resistance genes have evolved.

Regardless of the origins of MDR strains, it is important to understand the impact that solvent contamination of natural environments may have on the development of these strains. Contamination of soils, groundwater, and surface water bodies by petroleum fuel spills is common in industrialized nations (3). Limited resource availability and inadequate toxic waste control (3) increase the likelihood that contamination will occur in our own communities and the likelihood that we will come into close contact with MDR microbes. Rather than focus on artificially selected pure cultures of clinical isolates, we investigated the antibiotic resistance profiles of microorganisms inhabiting a natural system that includes an abundance of potential microbial competitors. The former organisms are subject to the stress inherent to modern antibiotic therapy, while the latter organisms are exposed to asphalt seeps containing high concentrations of organic solvents. Either way, the result remains the same: multiple-antibiotic-resistant microbes emerge. Thus, in nature there is more than one way to become resistant to antibiotics, and information regarding the acquisition of resistance genes may lead to more reasonable ways to combat them.

ACKNOWLEDGMENTS

This research was supported by a grant from the Nebraska Corn Board. T.H. was supported in part by the Dreyfus program, Department of Biochemistry, University of Nebraska, and by the Undergraduate Creative Activity and Research Experience (UCARE) Program, University of Nebraska.

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