Plasmid Frequency Fluctuations in Bacterial Populations from Chemically Stressed Soil Communities

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The frequency of plasmids in chemically stressed bacterial populations was investigated by individually adding various concentration of kanamycin, ampicillin, and mercuric chloride to soil samples. Viable bacterial populations were enumerated, soil respiration was monitored for up to 6 weeks as an indicator of physiological stress, and bacterial isolates from stressed and control soils were screened for the presence of plasmids. Low levels of the chemical stress factors did not for the most part significantly alter population viability, soil respiration, or plasmid frequency. Exposure to high stress levels of mercury and ampicillin, however, resulted in altered numbers of viable organisms, soil respiration, and plasmid frequency. Plasmid frequency increased in response to ampicillin exposure but was not significantly changed after exposure to kanamycin. In mercuric chloride-stressed soils, there was a decrease in plasmid frequency despite an increase in overall mercury resistance of the isolates, suggesting that mercury resistance in these populations is largely, if not completely, chromosome encoded. Chemical stress did not cause an increase in plasmid-mediated multiple resistance. A genetic response (change in plasmid frequency) was not found unless a physiological (phenotypic) response (change in viable cells and respiratory activity) was also observed. The results indicate that a change in plasmid frequency is dependent on both the amount and type of chemical stress.

The prospect of releasing genetically engineered microorganisms into the environment has heightened interest in the study of plasmid frequency and the rates of plasmid transfer in both pristine and stressed or disturbed environments (28). A major concern is that plasmids will mediate the transfer of genes within environmental samples (4, 11, 26, 33). Various investigators have studied the frequency of plasmids in bacterial populations exposed to physical or chemical stress, generally finding that bacteria isolated from environments where temperature, nutritional, or pollutant stresses prevail exhibit a high frequency of plasmids (20–22). For example, Hada and Sizemore (14) found a greater frequency of plasmid-containing organisms in an oil field (35%) than in a similar control site with no oil pollution (23%); most of the plasmids in the bacteria from the polluted site were 10 megadaltons or smaller, and approximately half of the strains had multiple plasmids. Large catabolic plasmids that also conferred mercury resistance have been found in soil bacteria exposed to halogenated alkanoic acids (15). Glassman and McNicol (12) found that 46% of estuarine heterotrophs in Chesapeake Bay, an area known to receive various pollutant stresses, carry plasmids; they reported that bacteria from relatively unpolluted sites had smaller plasmids (ca. 3 megadaltons) than those from more polluted sites (ca. 30-megadalton plasmids) and that bacteria from polluted sites tended to have multiple plasmids. Burton et al. (7) found a higher incidence of plasmids among *Pseudomonas*-like organisms in an industrially polluted river (18%) than in a nonpolluted upstream area (7%). Rolland et al. (30) found that antibiotic resistance plasmids were much more abundant in the intestinal bacterial biota of baboons ingesting human refuse than in those with little human contact.

These studies, which focused on environments in which stresses had been extant for unspecified, sometimes unknown, periods of time, have given a general picture that

plasmid frequency increases in populations exposed to chemical stress. It is of interest to determine the immediate effects of chemical stress on the frequency of plasmid DNA as well as the minimal stress needed for bacterial populations to respond genetically to a particular selective pressure. The present study attempts to assess the short-term effects of environmental stress on the frequency of plasmids within the bacterial populations of a soil microbial community by exposing the soil to several different chemical stress factors at low and high stress levels. The stresses selected were the antibiotics ampicillin and kanamycin and the heavy metal-containing compound mercuric chloride. The study examined both the physiological (respiration) and the genetic (plasmid frequency) responses of the bacterial populations within the chemically stressed soil communities.

MATERIALS AND METHODS

Soils. A silt loam soil (top 5.0 cm) collected from a grass-covered field in Louisville, Kentucky, was used for this study. The soil was passed through a sieve (1.7-mm mesh) to remove large pieces of debris and vegetation. The water-holding capacity of the soil and the water content of the soil were determined by standard methods (5).

Chemical stress factors. Stock solutions of chemical stress factors were prepared as follows: ampicillin trihydrate was dissolved in 0.02 N NaOH to a final concentration of 8.0 mg/ml; kanamycin sulfate and mercuric chloride were dissolved in distilled water to give final concentrations of 20.0 and 1.25 mg/ml, respectively. All stock solutions were filtered through 0.2-μm-pore-size filters (Millipore) into sterile tubes and stored in the dark at −20°C.

Low stress levels were selected by replica-plating 100 randomly selected soil isolates onto 0.1 strength Trypticase soy agar (BBL Microbiology Systems) plates containing incremental amounts of each of these chemical stress factors and determining the levels at which at least 60% of the test

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organisms survived. Based on these tests, 3.0 μg of mercuric chloride, 6.0 μg of kanamycin, and 25 μg of ampicillin per ml were added to soils for low-stress-level studies. It should be noted that these determinations were made on agar plates and not in soils, where adsorption to soil particles might lessen the toxicity of the stress factor. In high-stress-level tests, 50, 200, and 200 μg of mercuric chloride, kanamycin, and ampicillin per ml, respectively, were added to soils.

Physiological stress and soil respiration. Fifty-gram subsamples of soil were distributed to biometer flasks (Bellco, Vineland, N.J.). Each chemical was dissolved in sterile distilled water and added to each flask to achieve the appropriate concentration of that stress factor and a 60% water-holding capacity level. Flasks were incubated in the dark at 28°C, and CO_2 evolved was measured every 1 to 3 days by the method of Bartha and Pramer (3). Total carbon dioxide evolved (millimoles per 50 grams of soil [wet weight]) was calculated as a measure of soil microbial respiration, which was used as a measure of physiological response of the soil microbial community to the various stress factors.

Viable heterotrophic counts. For determining total numbers of viable heterotrophic bacteria, 1-g soil subsamples were collected from each flask after 28 days of incubation. Samples were diluted in 0.1 M phosphate buffer, pH 7.0 (8), and spread plated onto 0.1 strength Trypticase soy agar for enumeration. Plates were incubated at 25°C for up to 2 weeks, after which numbers of colony-forming bacteria (viable heterotrophs) were counted.

Resistance and multiple resistance after exposure to stress factors. Isolates from soils exposed to high levels of stress (i.e. 50, 200, and 200 μg of mercuric chloride, kanamycin, and ampicillin per ml, respectively) were replica-plated onto 0.1 strength Trypticase soy agar containing 25, 100, and 100 μg of mercuric chloride, kanamycin, and ampicillin per ml, respectively. Isolates were defined as resistant to the particular stress if they exhibited growth on the plate containing the stress factor. These tests were used to define organisms with both single and multiple resistance traits.

Plasmid screening. Colonies from enumeration plates were randomly picked and streaked onto 0.1 strength Trypticase soy agar for isolation. After restreaking for purification, isolates were grown in 0.1 strength Trypticase soy broth or on solid agar plates if broth culture yielded inadequate densities of cells; isolates grown on solid medium were aseptically scraped from plates and suspended in sterile distilled water. Cell suspensions were spun for 5 min in a Brinkman microcentrifuge, the supernatant was discarded, and the cell pellet was saved for plasmid screening.

Plasmid isolation was performed by a modified Kieser procedure (19); this method is effective for plasmid recovery from both gram-positive and gram-negative bacterial cells. Cell pellets were suspended in 0.5 ml of lysozyme solution (2.0 mg/ml) and incubated for 30 min at 37°C. NaOH-sodium dodecyl sulfate (250 μl) was added, and the isolates were incubated at 55°C for 30 min and cooled to room temperature. Acid phenol-chloroform (80 μl) was added to the lysates, which were then vortexed to mix the phases and centrifuged at 10,000 rpm for 2 min. The upper aqueous phase (35 μl) was added to gel slots for electrophoresis. Electrophoresis was carried out on a horizontal 0.7% agarose gel. Plasmids pBR322 (3.9 kilobases [kb]) and pGR71 (8.4 kb), propagated in *Escherichia coli* RR1 and *E. coli* HB101, respectively, were used as positive controls. A plasmidless strain of *E. coli* was used as a negative control.
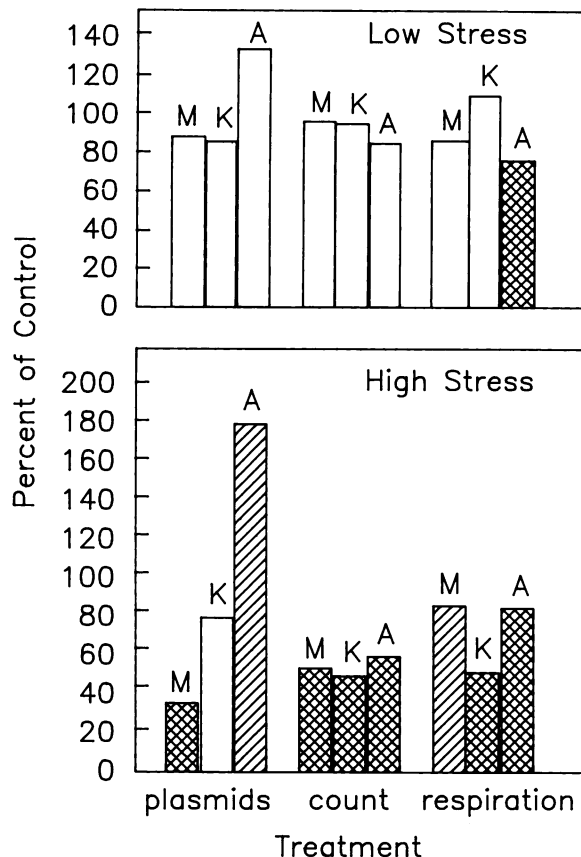

FIG. 1. Histograms showing effects of low and high levels of soil stress factors on plasmid frequency, viable count, and soil respiration. Results are shown as percent of control value. Crosshatched bars indicate statistically significant difference from control. Hatched bars indicate that the difference from the control approached statistical significance. Concentrations of chemical stress factors used in the low-stress tests were: mercuric chloride (M), 3 μg/ml; kanamycin (K), 6 μg/ml; ampicillin (A), 25 μg/ml. Concentrations of chemical stress factors used in the high-stress tests were: mercuric chloride, 50 μg/ml; kanamycin, 200 μg/ml; ampicillin, 200 μg/ml.

Gels were stained in Tris-borate buffer (25) containing ethidium bromide (0.5 μg/ml) for 30 min and viewed on a UV transilluminator at a wavelength of 260 nm.

Statistical analyses. Statistical analyses were performed by using the Statistix and Stats Plus computer programs. Respiration and viable count data were statistically analyzed by using t tests for comparison of pairs of groups. The t tests were performed as described by Rosner (31). A P value of 0.05 was used as the discriminator of statistical significance. Plasmid frequency and posttreatment resistance data were statistically analyzed with a one-sided statistical test for the difference between two population proportions as described by Goodwin and Kemp (13) and used in similar studies by Burton et al. (7). When small sample sizes did not allow the use of the test for the difference between two population proportions, a one-sided Fisher's exact test was performed (31).

RESULTS

Viable counts. The number of viable heterotrophs in the unstressed control soils used in these experiments was

approximately 2×10^7 CFU/g (dry weight) of soil. All of the high-level treatments but none of the low-level treatments resulted in significantly reduced numbers of viable cells compared with the controls (Fig. 1).

Physiological response—soil respiration. Although the low-level stress treatments were not lethal to most soil microorganisms, there were detectable changes in soil respiration in response to low levels of all three stress factors (Fig. 1). Only the soil treated with ampicillin, however, had a significantly lower rate of CO_2 evolution than the unstressed control ($P = 0.04$).

In the higher-stress-level tests, more carbon dioxide was produced in the unstressed control than in any of the stressed samples (Fig. 1). The kanamycin (200 μg/ml) and ampicillin (200 μg/ml) treatments were significantly different from the controls in terms of CO_2 evolution ($P < 0.001$ and $P = 0.02$, respectively). The mercuric chloride (50 μg/ml) treatment approached statistical significance ($P = 0.082$).

Plasmid frequency. The low-level-stress tests showed that the overall plasmid frequency for the 6-week period (defined as percentage of isolates containing plasmids) was 24.1% (14 of 58) for the unstressed control. There was no significant difference in plasmid frequency in any of the stress treatments compared with the controls (Fig. 1). Populations from kanamycin-, mercuric chloride-, and ampicillin-stressed soils exhibited plasmid frequencies of 20.3% (12 of 59), 21.7% (15 of 69), and 32.2% (19 of 59), respectively.

Replicate samples from all the high-stress treatments were statistically homogeneous, which allowed the pooling of individual results for subsequent statistical comparisons between treatments. The plasmid frequency for the unstressed control in the high-level-stress tests was 17.3% (9 of 52). The soil treated with mercuric chloride had a plasmid frequency of 6.2% (3 of 48), which was significantly lower than the control (Fig. 1). The soil treated with kanamycin had a plasmid frequency of 13.7% (7 of 51), which also was lower than the control but not at a statistically significant level. The soil treated with 200 μg of ampicillin per ml exhibited a plasmid frequency of 31.0% (14 of 45), which was higher than that of the control and approached statistical significance ($P = 0.086$). Thus, exposure to mercury resulted in lower plasmid frequency, kanamycin caused little change in plasmid frequency, and ampicillin appeared to increase plasmid frequency.

Posttreatment resistance. The percentage of kanamycin-resistant cells isolated from the kanamycin-treated soil (6.7%) was less than that found in untreated controls (14.3%). Likewise, the percentage of isolates resistant to 100 μg of ampicillin per ml in ampicillin-treated soils (60.7%) was less than that from untreated controls (69.4%). Neither difference was statistically significant at the 0.05 level. After treatment with mercuric chloride, the percentage of isolates resistant to mercury was more than fourfold higher in mercuric chloride-treated soils (17.1%) than in untreated controls (4.1%). This increase approached statistical significance ($P = 0.052$). However, it appears that this increase in mercury resistance is not plasmid mediated, because none of the nine plasmid-containing isolates from mercury-treated soils and none of the nine plasmid-containing isolates from untreated soils were resistant to $HgCl_2$ (25 μg/ml), whereas 19% (6 of 32) of the plasmidless strains from mercury-treated soil and 5% (2 of 40) of the plasmidless isolates from untreated soils exhibited resistance to mercury.

Posttreatment multiple resistance. Most strains showed no multiple resistance. None were resistant to all three factors, and of the plasmid-bearing strains exposed to the stress

factors, only one showed multiple resistance to two factors; this strain, which had been exposed to mercury, was resistant to ampicillin and kanamycin but not mercury. Among the plasmidless strains, exposure to mercury resulted in a threefold increase, from 5 to 15%, in strains that were resistant to both mercury and ampicillin. This was the only instance in which exposure to a stress factor appeared to lead to increased multiple resistance. Aside from these instances, multiple resistance occurred in 0% of the isolates from soils exposed to stress factors. Thus, in most cases chemical stress selected against rather than for multiple resistance in these soil bacterial populations.

DISCUSSION

An important question addressed in this study is whether exposure of soil to chemical stress for a relatively short period of time will cause an increase in the frequency of plasmids carried by the bacterial populations in that stressed community. Some studies have indicated that increased plasmid frequency, e.g., for antibiotic resistance, can occur without stress (17, 27), whereas others have found that plasmid frequency increase is a response to stress (7, 15, 18). Increased plasmid frequencies have been reported in response to physical and chemical stress and have been associated with increased resistance (16, 18, 23). Several previous studies that examined the incidence of plasmids or the amount of bacterial resistance to a particular chemical at an already chemically polluted site that had been exposed to chemical pollutants for up to many years in some cases found that stress due to pollution results in increased plasmid frequency and associated metal and antibiotic resistance (7, 9, 14).

In many cases (20–22), exposure to a chemical stress has been reported to cause an increase in multiple resistance. However, in the current study, exposure of soil populations to chemical stress did not result in increased multiple resistance in plasmid-bearing strains. In fact, chemical stress appeared to select against multiple resistance except in the case of mercury-stressed plasmidless strains, in which chromosomally mediated mercury and ampicillin resistance increased. Mercury resistance in penicillinase-producing bacteria has been described (32).

The low levels of the chemical stress factors used in the current study had negligible effects on the physiological and genetic status of the soil bacterial populations, with only ampicillin causing a significant decrease in overall soil respiration. None of the low-level stress factors caused a significant change in plasmid frequency. The frequency of plasmids in the bacterial populations treated with the high level of ampicillin was almost twice the plasmid frequency found in the unstressed control; this increase approached statistical significance and suggests that the moderate increase in the frequency of plasmids found in the low-level ampicillin-treated soil may have been a real response to ampicillin stress. Although the kanamycin-treated soil also had a significantly reduced level of CO_2 evolution, and even though kanamycin resistance is known to be plasmid mediated (6), there was no significant change in the frequency of plasmids in response to the high level of kanamycin. It is not clear why there was no increase in overall resistance to ampicillin in the ampicillin-treated soil even though there was an increase in plasmid frequency.

Unlike the increased plasmid frequency that occurred in response to ampicillin treatment, exposure to the high level of mercury resulted in decreased plasmid frequency despite

the overall increase in mercury resistance within the microbial community. Barkay and Olson (2) previously reported that there was a high correlation between mercury contamination of sediments and the occurrence of the *mer* gene, which codes for mercury volatilization, but that other mechanisms of resistance may partially determine the presence of mercury-resistant bacteria. Barkay recently described an alternative mechanism for mercury resistance that is not encoded in the *mer* gene (1). Although mercury resistance is often encoded by plasmids (10, 29, 32), Mahler et al. (24) recently isolated several mercury-resistant gram-positive bacilli in which mercury resistance was not plasmid encoded. A similar resistance mechanism may have occurred in the mercury-resistant isolates recovered from the mercury-stressed soils in this study.

The importance of the type of stress employed should be dependent on the type of resistance that plasmids already present in the soil bacteria encode at the time of sampling. If there are no plasmids conferring resistance to a certain chemical stress, raising the level of that stress might only serve to decrease the number of bacteria which carry plasmids. If those plasmids were not selected for or against, the proportion of plasmid-bearing bacteria would remain the same; this could explain the results obtained with the kanamycin-treated soil, in which no change in plasmid frequency was observed. An increase in plasmid frequency, as observed for the ampicillin-treated soils, almost certainly represents enrichment for plasmids carrying the genes for ampicillin resistance. A decrease in plasmid frequency, as observed in the mercuric chloride-treated samples, may be attributable to excessive stress placed on a bacterium carrying a nonessential plasmid in the presence of high levels of chemical stress. Warnes and Stephenson (34) have found that excessive plasmids are unstable and lost from bacterial populations. The increase in mercuric chloride-resistant isolates in this study was presumably due to chromosome-encoded resistance. If there were relatively few plasmids conferring resistance to mercuric chloride in the soil initially, then the stress might have caused a reduction in the number of plasmids which coded for traits other than mercury resistance due to the increased metabolic strain imposed by a chemical stress and maintenance of a nonessential plasmid at the same time. This could select against those organisms carrying plasmids coding for processes other than mercuric chloride resistance.

In conclusion, contrary to the predicted response, only ampicillin exposure resulted in increased plasmid frequency; other stress factors caused little change or a decrease in plasmid frequency. Thus, it appears that changes in plasmid frequency in response to chemical stress factors are dependent on both the amount and type of chemical stress.

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