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Genetic Variation within a Lotic Population of *Janthinobacterium lividum*

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An understanding of the genetic variation within and between populations should allow scientists to address many problems, including those associated with endangered species and the release of genetically modified organisms into the environment. With respect to microorganisms, the release of genetically engineered microorganisms is likely to increase dramatically given the current growth in the bioremediation industry. In this study, genetic variation within a lotic, bacterial population of *Janthinobacterium lividum* was measured with restriction fragment length polymorphism analysis. Chromosomal DNA from 10 Kettle Creek (Hawk Mountain Sanctuary, Kempton, Pa.) *J. lividum* isolates was digested with six restriction endonucleases and probed with a 7.5-kb pKK3535 fragment containing the *E. coli rrnB* rRNA operon. Genetic variation, as measured in terms of nucleotide diversity, was high within the population. The 0.0781 value for genetic variation was especially high given the conservative nature of the genetic probe. The average percent similarity among isolates within the population was 67.25%. Pairwise comparisons of nucleotide diversity values (π) and similarity coefficients (F) yielded values ranging from 0.0032 to 0.1816 and 0.3363 to 0.9808, respectively. Putative clonemates were not present within the group of isolates; however, all isolates shared 14 fragments across a spectrum of six restriction enzymes. The presence of these common fragments indicates that restriction fragment length polymorphism analysis may provide population- or species-specific diagnostic markers for *J. lividum*. Data that suggest a plume effect with respect to the downstream movement of *J. lividum* are also presented. An increase in genetic variation within groups of isolates along the longitudinal gradient of Kettle Creek is also suggested.

Although many microbial studies have characterized genetic variation at the species level (8, 19, 27), fewer studies have addressed genetic variation at the level of the population (24). Intrapopulation genetic variation and gene flow between populations become especially important when endangered species are being considered. The fate of an endangered species sometimes reflects the genetic variation within a single population (16). The welfare of the population is also sometimes dependent upon the immigration of individuals into the population (gene flow) (22, 37). The more genetic variation within a population, the greater the potential for adaptation to environmental changes (2). More studies in the area of intrapopulation genetic variation of natural populations are necessary before we can better serve the needs of endangered species.

Intrapopulation genetic variation and gene flow are also important considerations when one is faced with the task of predicting the environmental risks associated with the release of genetically modified organisms. Understanding the genetics of the recipient population and being able to predict the movement of this genetic material allows one to better estimate the impact of genetically modified organisms (45). The ecological consequences of genetically modified organisms are not always predictable, however (11). Genetically engineered microorganisms (3, 15, 36) will likely become common in the future; it is our responsibility to predict the consequences of their release.

In line with this responsibility, we selected *Janthinobacterium lividum* for our study of intrapopulation genetic variation. *J. lividum*, which was previously classified as *Chromobacterium lividum*, is a purple bacterium that is common in soil and water in temperate regions (39). In studying 10 bacterial species collected from 50 streams in eastern Pennsylvania, we found that *J. lividum* was present in the greatest number of streams (18). Because of its broad geographic distribution, *J. lividum* appears to be an ideal species for studying intrapopulation genetic variation, interpopulation genetic variation, and gene flow.

Several approaches are available for estimating genetic variation. Multilocus enzyme electrophoresis (12, 35) and restriction fragment length polymorphism (RFLP) analysis (23, 29) are commonly used. A recent addition to the collection of approaches is the random amplified polymorphic DNA technique (17). We used RFLP analysis to study genetic variation within a *J. lividum* population. RFLP analysis has been used to study genetic variation within several bacterial taxa, including *Staphylococcus aureus* (13), *Legionella* species (34), *Pseudomonas solanacearum* (8), and *Clavibacter michiganense* subsp. *sepedonicum* (27). RFLP analysis is a sensitive technique that surveys genomes for sequence changes detected as the addition or deletion of restriction endonuclease recognition sites. Restriction fragment patterns generated by digestion of DNA with restriction endonucleases, separation of fragments by gel electrophoresis, and hybridization with a genetic probe can be analyzed to provide statistical estimates of genetic variation (29).

Using RFLP analysis of genes encoding rRNA, we demonstrate that the *J. lividum* population exhibits a high degree of genetic variation. We also show that RFLP analysis may provide molecular markers for *J. lividum*. In addition, there is some evidence for genetic patterns along the longitudinal gradient of the stream.

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MATERIALS AND METHODS

Isolation of *J. lividum*. Multiple water samples were collected from 12 sites along a 3-km stretch of Kettle Creek (Hawk Mountain Sanctuary, Kempton, Pa.). Kettle Creek is a pristine, first-order stream which, along a portion of its length, flows beneath a boulder field (River of Rocks). Stream water (100 μl) was plated onto peptone yeast extract agar (PYE; 0.5% BactoPeptone, 0.1% yeast extract, 1.5% agar [pH 7.0]). Potential *J. lividum* colonies were isolated and cultured at room temperature (22°C). The taxon was initially identified by fatty acid gas chromatography (Microcheck, Inc.) as *J. lividum*. Other isolates were subsequently identified as *J. lividum* by using the following screening method.

J. lividum, a strict aerobe that produces the pigment violacein, is resistant to benzylpenicillin, does not grow at 37°C, and produces acid from the fermentation of the carbohydrate xylose but not from trehalose (10, 38, 39). Potential *J. lividum* isolates were tested for growth on PYE supplemented with 10 μg of benzylpenicillin per ml and on PYE plates at 37°C for 4 days. Anaerobic growth was tested by placing PYE plates containing the isolates into a GasPak jar for 7 days. The ability to ferment trehalose and xylose was examined by growing isolates in Hugh and Leifson medium without agar (0.2 g of peptone, 0.5 g of NaCl, 0.3 g of K_2HPO_4, 0.003 g of bromothymol blue, 1 g of trehalose or xylose, water to 100 ml) for 7 days (38). The presence of violacein was tested spectrophotometrically by methods described by Sneath (38).

Isolation of chromosomal DNA. Chromosomal DNA was isolated from the 10 *J. lividum* isolates by using a modified version of a protocol described by Zyskind and Bernstein (46). Overnight cultures of *J. lividum* cells (1.2 ml) in PYE broth were harvested by centrifugation at 13,446 × *g* for 15 s, and this process was repeated. The pellet was resuspended in 1 ml of lysis buffer (10 mM Tris base, 1 mM Na_2EDTA, 1 M sucrose, 10 mg of lysozyme per ml [pH 8.0]) and was incubated for 1 h at 37°C. The cells were centrifuged for 10 min at 13,446 × *g* at 4°C and were resuspended in 310 μl of HTE buffer (50 mM Tris base, 20 mM Na_2EDTA [pH 8.0]). A 2% *N*-lauroylsarcosine–sodium salt solution (350 μl) was added, and the tube was inverted three to five times. Five microliters of RNase A (10 mg/ml) in TE (10 mM Tris base, 1 mM Na_2EDTA [pH 8.0]) was added and incubated at 37°C for 15 min. Then 35 μl of 10-mg/ml proteinase K in TNE buffer (10 mM Tris base, 10 mM NaCl, 0.1 mM Na_2EDTA [pH 8.0]) was added and incubated at 50°C for 1 h. The lysate was mixed vigorously for 2 min. Five hundred microliters of the lysate was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) with Phase Gel Lock tubes (5 Prime → 3 Prime, Inc.). Fifty microliters of 3.0 M sodium acetate (pH 5.2) was added to the aqueous DNA solution and mixed. Isopropanol (500 μl) at −20°C was added, and the tube was inverted and stored at −20°C for 20 min to allow the DNA to precipitate. The DNA was pelleted by centrifugation at 13,446 × *g* at 4°C for 20 min. The pellet was washed three times with 1 ml of 70% ethanol (−20°C) and was centrifuged at 13,446 × *g* at 4°C for 5 min. The pellet was dried on the bench top and was dissolved in 25 μl of TE buffer. The DNA concentration was estimated by the Saran Wrap method described by Sambrook et al. (33).

Digestion of chromosomal DNA and separation of fragments. Six restriction digests were performed for each isolate by using *Hin*dIII, *Eco*RI, *Pst*I, *Nsi*I, *Sma*I, and *Bam*HI (United States Biochemical Corporation, New England Biolabs, Inc.). One microgram of DNA was digested according to the manufacturer's instructions for 3 to 5 h. Restriction fragments were separated by size by gel electrophoresis in 0.8% agarose and 0.5× TBE (0.045 M Tris-borate, 0.001 M Na_2EDTA [pH 8.0]) for 8 h. Agarose gels were stained in 1 μg of ethidium bromide per ml for 25 min and were photographed.

Preparation of hybridization probe. Plasmid pKK3535 (5, 6) was isolated from *Escherichia coli* HB101 by an alkaline lysis procedure described by Morelle (28). pKK3535 was digested with *Bam*HI (United States Biochemical Corporation), and the fragments were separated by electrophoresis in 0.8% agarose and 1× TAE (0.04 M Tris, 0.02 M sodium acetate, 0.0025 M Na_2EDTA [pH 8.3]). The 7.5-kb fragment containing the entire *E. coli rrnB* rRNA operon was purified from the agarose gel with the Gene Clean II Kit (Bio 101, Inc.). One hundred nanograms of purified 7.5-kb fragment was labeled with biotin with the Random Primed Images Biotin Labeling Kit (United States Biochemical Corporation) according to the manufacturer's instructions. DNA was precipitated in 0.15 M NaCl and 100% ethanol as described in Sambrook et al. (33). The DNA concentration was estimated by the Saran Wrap method (33).

Southern blotting and Southern hybridization. Digested DNA was transferred from the agarose gel to a Gene Images membrane (United States Biochemical Corporation) according to the manufacturer's instructions. Prehybridization, hybridization, and posthybridization washes were also performed according to the manufacturer's instructions. Membranes were prehybridized in hybridization solution (50% formamide, 6× SSPE [0.9 M NaCl, 60 mM NaH_2PO_4, 6 mM Na_2EDTA {pH 7.4}], 5× Denhardt's solution [5 Prime → 3 Prime, Inc.] 0.5% sodium dodecyl sulfate [SDS], and 200 μg of denatured, sheared salmon sperm DNA [5 Prime → 3 Prime, Inc.] per ml) for 4 h at 45°C. Hybridizations of 100 ng of denatured, biotinylated probe were conducted in hybridization solution supplemented with 10% dextran sulfate for 18 h at 45°C. Membranes were washed twice with 5 ml of 2× SSC (0.3 M NaCl, 30 mM sodium citrate [pH 7.4])–0.5% SDS per cm^2 for 5 and 20 min at room temperature, were washed twice with 5 ml of 0.1× SSC–0.1% SDS per cm^2 for 30 min each at 50°C, and were washed once with 2× SSC for 5 min at room temperature. Hybridized DNA was detected with the Gene Images Non-Isotopic Nucleic Acid Detection System (United States Biochemical Corporation) according to manufacturer's protocol. Membranes were exposed to Kodak X-Omat RP film and developed. Multiple exposures, commonly 15 min, 30 min, 1 h, and 2 h, were taken to ensure proper identification of bands.

Data analysis. Restriction fragment patterns from the 10 *J. lividum* isolates were compared pairwise for the presence or absence of restriction fragments. All bands, regardless of size and intensity, were recorded by visual inspection. The proportion of shared fragments (F), termed the similarity coefficient, was calculated as $\hat{F} = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of common fragments between isolate x and isolate y, n_x is the number of fragments from isolate x, and n_y is the number of fragments from isolate y (29). For each pair of isolates, F values were calculated from the number of common fragments and the total number of fragments generated by all six restriction endonucleases. Nucleotide diversity (π), the average number of nucleotide differences between isolates, was calculated as $\hat{\pi} = [(-\ln \hat{F})/r]$, where r is the number of nucleotide base pairs in the restriction endonuclease recognition site (29). All restriction endonucleases used in this study to digest genomic DNA recognize sites of

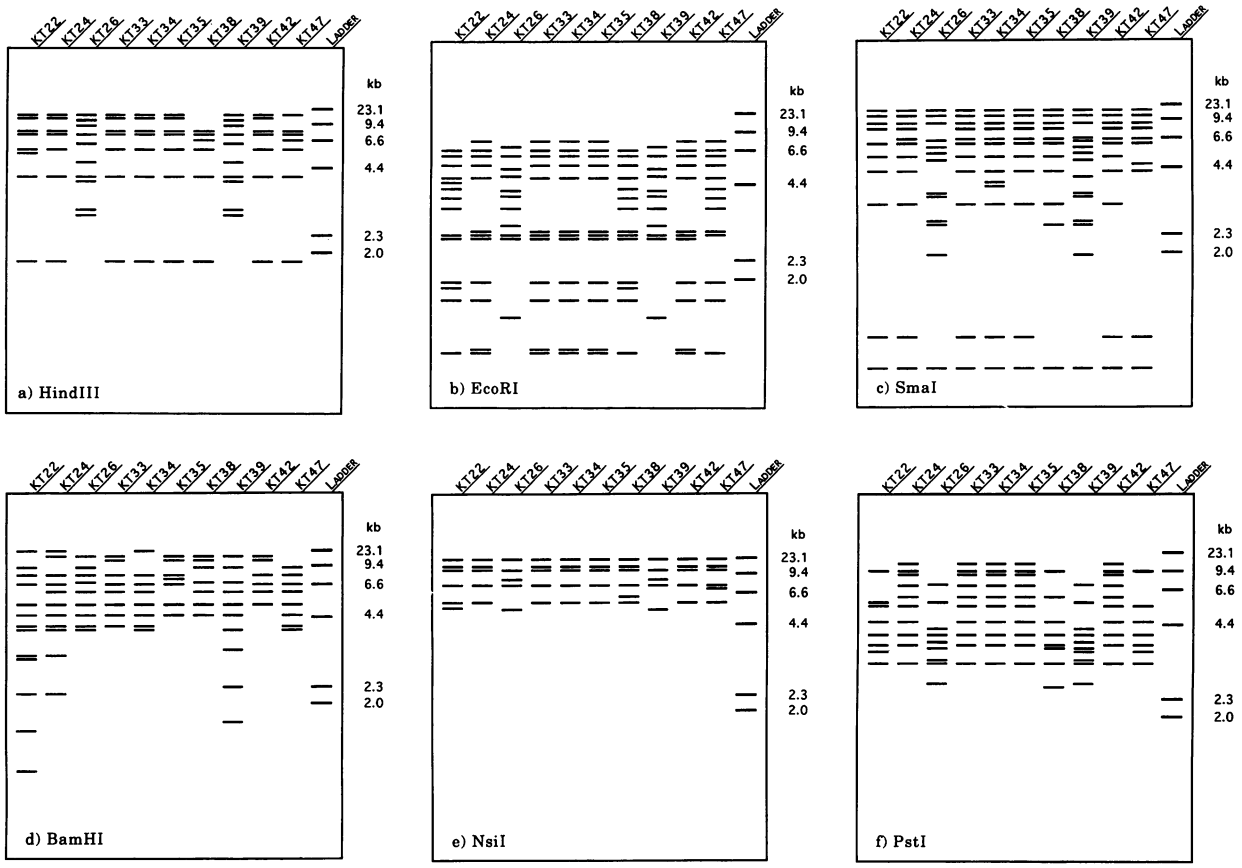

FIG. 1. Schematic representations of RFLP analyses of restriction endonuclease-digested chromosomal DNA from 10 *J. lividum* isolates hybridized to the 7.5-kb *Bam*HI pKK3535 fragment. Specific restriction endonucleases are noted on each panel. Photobiotinylated *Hin*dIII-digested lambda DNA was used as a ladder. The two smallest fragments of the ladder, 0.6 and 0.1 kb, were not visible on any of the Southern blots. The RFLP patterns shown here are representations of bands from multiple film exposures.

$r = 6$. Intrapopulation genetic variation is calculated as the average nucleotide diversity within the population (29). An unweighted pair group method using arithmetic averages dendrogram of nucleotide diversities was constructed by the method of Sneath and Sokal (40).

RESULTS

J. lividum appears to exhibit a widespread distribution throughout eastern Pennsylvania. Fifty streams were sampled within a 16,000-km^2 area, and *J. lividum* was collected from 25 of these streams. Its broad distribution makes this species a good candidate for future studies of interpopulation genetic variation and gene flow. The current study of intrapopulation genetic variation, in part, was designed to further test the adequacy of *J. lividum* for future studies. We were in search of a bacterial species which exhibits a high degree of genetic polymorphism and has readily detectable genetic markers. These characteristics were found in *J. lividum* and will facilitate the analysis of a *J. lividum* metapopulation, i.e., a group of interacting populations (26).

Macroecologists define the term population as a group of coexisting individuals that belong to the same species; that is, they function as a unit both evolutionarily and ecologically (32). Microbiologists commonly define a population as a group (a clone) of cells derived from a single microbial cell (1, 4). In our study, we consider the sum total of all *J. lividum* clones present in Kettle Creek as a population. Within Kettle Creek, selection pressures differentially affect different genetic types within this population. Other populations of *J. lividum* exist in other aquatic ecosystems—for example, in the other 24 streams from which we collected this species. If gene flow occurs among these populations, then one or more *J. lividum* metapopulations exist in eastern Pennsylvania. The discrete nature of different bacterial populations is reinforced by working with populations from lotic ecosystems. We took the isolation of coexisting groups one step further by designating our population as one within the limits of a first-order stream; first-order streams are small headwater streams without tributaries. An input of new genetic types from tributaries therefore does not introduce complexity into our current set of data. This complexity will be addressed in the future.

The *rrnB* rRNA genetic probe effectively identified polymorphisms among our *J. lividum* isolates. Given the conservative nature of the probe (9, 20, 34), there was a potential for limited polymorphism. Each one of the six restriction enzymes exhibited differences in restriction patterns among the 10 isolates (Fig. 1). Within the range of fragment sizes from 700 to 23,000 bp, 5 to 14 distinct bands were present per isolate for each restriction enzyme. Fragment sizes were determined through third-order polynomial curve fitting of the 1/kb versus migration distance relationship. The inverse of the fragment size was based on the *Hin*dIII-digested

TABLE 1. Nucleotide diversity ($\hat{\pi}$) values and similarity coefficients ($\hat{F}$) for *J. lividum* isolates

Isolate	Nucleotide diversity value or similarity coefficient[a] for isolate:									
	KT22	KT24	KT26	KT33	KT34	KT35	KT38	KT39	KT42	KT47
KT22		0.0372	0.1542	0.0479	0.0431	0.0505	0.0546	0.1672	0.0531	0.0372
KT24	0.8000		0.1572	0.0078	0.0077	0.0128	0.0500	0.1708	0.0113	0.0364
KT26	0.3966	0.3894		0.1605	0.1635	0.1756	0.1438	0.0117	0.1741	0.1497
KT33	0.7500	0.9541	0.3818		0.0095	0.0048	0.0412	0.1746	0.0032	0.0356
KT34	0.7719	0.9550	0.3750	0.9444		0.0146	0.0527	0.1775	0.0131	0.0348
KT35	0.7387	0.9259	0.3486	0.9714	0.9159		0.0437	0.1816	0.0049	0.0421
KT38	0.7207	0.7407	0.4220	0.7810	0.7290	0.7692		0.1427	0.0421	0.0421
KT39	0.3667	0.3590	0.9322	0.3509	0.3448	0.3363	0.4248		0.1802	0.1557
KT42	0.7273	0.9346	0.3519	0.9808	0.9245	0.9709	0.7767	0.3393		0.0405
KT47	0.8000	0.8037	0.4074	0.8077	0.8113	0.7767	0.7767	0.3929	0.7843	

[a] Values above the diagonal are $\hat{\pi}$ values; those below the diagonal are $\hat{F}$ values.

lambda DNA ladder (United States Biochemical Corporation). RFLP analysis identified 14 bands common to all *J. lividum* isolates. Common bands and their respective restriction enzymes include 3.9 and 7.3 kb (*Hin*dIII); 2.8 and 6.0 kb (*Eco*RI); 0.8, 9.1, 11.6, and 14.8 kb (*Sma*I); 4.9 kb (*Bam*HI); 7.4, 11.3, and 20.5 kb (*Nsi*I); and 3.1 and 3.9 kb (*Pst*I). These bands may represent population- or species-specific diagnostic markers for *J. lividum*.

Intrapopulation genetic variation was high and had a value of 0.0781. A collection of more isolates would likely yield an even higher value, indicating a very genetically diverse population. Within the population defined by the 10 isolates, nucleotide diversity ($\hat{\pi}$) ranged from 0.0032 to 0.1816 (Table 1). Similarity coefficients ($\hat{F}$) ranged from 0.3363 to 0.9808. Putative clonemates were not found, although isolates fell into four genetic clusters (groups I to IV) (Fig. 2). Even the three isolates (KT24, KT33, and KT42) collected from the same spring at the headwaters of Kettle Creek exhibited different RFLP patterns. One cluster of isolates, group I, composed of KT26 and KT39, was notably distinct ($\hat{\pi}$ = 0.1643) from the other three groups. Group II (KT22 and KT47), group III (KT24, KT33, KT34, KT35, and KT42), and group IV (KT38) were relatively similar in comparison with their dissimilarity with group I. Groups II, III, and IV clustered within a nucleotide diversity value of 0.0466. All four groups were separated by a nucleotide diversity value of at least 0.0421. Clusters do not necessarily represent taxonomic groups; they merely reflect clusters in the dendrogram. Future intrapopulation and interpopulation studies of *J. lividum* may provide more perspective on the genetic and ecological relevance of these clusters.

One indication of ecological relevance is that there appear to be genetic patterns along the longitudinal gradient of Kettle Creek (Fig. 3). At the stream's origin (site A), there is a pocket of genetically similar, although not identical, isolates. KT34 and KT35 were collected downstream from site A at site C. All of the isolates from sites A and C belong to the same genetic cluster, group III (Fig. 2). KT38 and KT47,

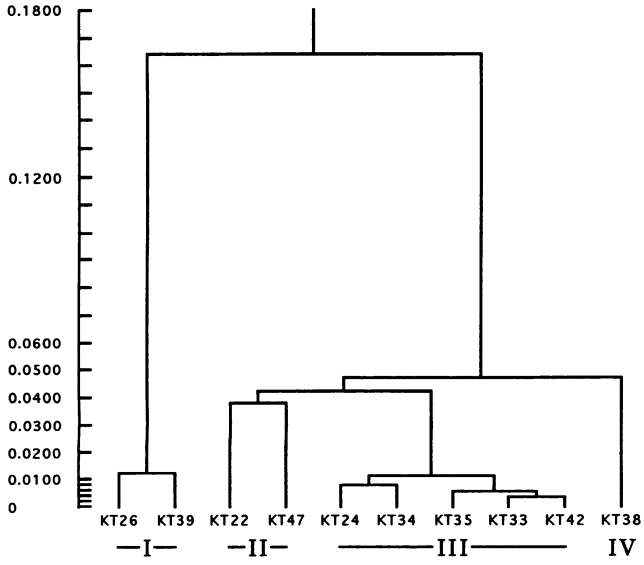

FIG. 2. Unweighted pair group method using arithmetic averages dendrogram of genetic relationships among the 10 *J. lividum* isolates. The vertical scale denotes nucleotide diversity values ($\hat{\pi}$). I, II, III, and IV designate genetic groups.

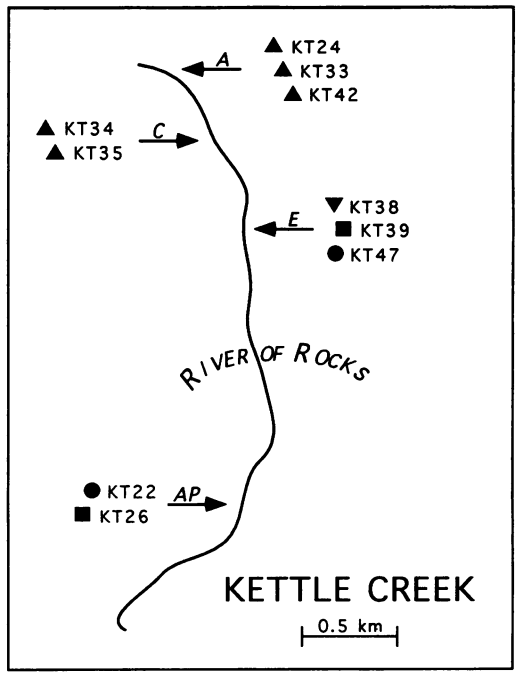

FIG. 3. Schematic representation of the headwaters of Kettle Creek. *A*, *C*, *E*, and *AP* denote collection sites. Symbols denote genetic groups: ■, group I; ●, group II; ▲, group III; ▼, group IV.

both of which were collected further downstream (site E), are members of the major cluster of groups II, III, and IV. KT39 of the more distant group I was also collected at site E. Representatives of both major clusters were collected downstream from the River of Rocks at site AP. The above data suggest, although not conclusively, that the longitudinal pattern reflects a gradient of overlapping bacterial plumes. The data also suggest that there is an increase in the genetic variation of *J. lividum* along the longitudinal gradient of Kettle Creek. Nucleotide diversity values among isolates increased from 0.0074 at site A to 0.0146 at site C; values at sites E and AP are 0.1135 and 0.1542, respectively.

DISCUSSION

J. lividum exhibits characteristics which make it an ideal species to use for studies related to endangered species and genetically engineered microorganisms. It has a broad distribution, which may conform to the structure of a metapopulation. A fair amount of work on the *J. lividum* rRNA cistron has been conducted (10). rRNAs are functionally homologous among species but carry sufficient sequence variability to discern evolutionary relationships among bacteria (4). The rRNA work conducted by De Ley et al. (10) and future RNA and DNA sequencing studies may contribute significantly to our understanding of the movements of bacteria within metapopulations. *J. lividum* also has natural mutant forms (18) which may provide insight into the spatial distribution of genetic types. High intrapopulation genetic variation and the presence of molecular markers, as noted in this study, would also assist in the study of bacterial populations, metapopulations, and the movement of bacteria to and from these natural groupings.

When dealing with the topics of endangered species and conservation biology, people normally do not think in terms of endangered bacteria. This may change in the future. Nonetheless, conservation biology needs better techniques for tracking the movement of members of many taxonomic groups. Bacterial studies may provide productive techniques or opportunities. For example, if the geographical distribution of genetic types is known for an enteric, bacterial species, then the movement of its host could be monitored. Availability of molecular markers and an understanding of the spatial layout of genetic types would contribute significantly to the assessment of risks associated with the release of genetically engineered microorganisms. An integral part of risk assessment is having the information to predict the movement of genetically modified organisms (45). Hadrys et al. (17) illustrated many applications of genetic markers, including the determination of interspecific gene flow and the hybridization of populations. Lu et al. (23) documented the use of molecular markers in identifying different populations of the fall armyworm moth. Several studies have identified genetic markers by using DNA encoding rRNA restriction analysis, which characterizes taxonomic relationships within and between species (e.g., *Lactococcus* species [31], *Staphylococcus* species [9, 41], *Staphylococcus aureus* [13], *Yersinia* species [30], *Legionella* species [34], *Streptococcus uberis*, and *Streptococcus parauberis* [43]).

Many studies of genetic variation within bacterial species are based on pooled collections of strains from widely separated geographic regions (e.g., see references 19, 27, and 44). If we are to access the impact of introducing genetically engineered microorganisms into the environment (3, 14, 15, 36), a more fundamental population (deme) approach should be adopted. For example, Istock et al. (21) studied a population of *Bacillus subtilis* collected from a 200-cm^3 microsite in a desert. Masters et al. (24) studied *Deinococcus radiopugnans* isolated from soil adjacent to a lake. They found extraordinary genetic variation, which they could not attribute to geographical differences among isolates. The high degree of variation in our *J. lividum* population also cannot be attributed to geographical differences; our isolates were collected within 3 km of one another. McArthur et al. (25) suggest that soil bacteria may be genetically more diverse than those with specific hosts or those with less-variable environments (e.g., intestine), because there appears to be a positive correlation between genetic diversity and habitat variability. This may also be true for aquatic populations. Extensive genetic recombination may also explain high levels of genetic variation within local populations. Istock et al. (21) suggest that genetic recombination within bacterial populations may be more common than once thought. The lack of putative clonemates within our samples and the high degree of genetic variation within the *J. lividum* population may reflect extensive genetic recombination.

Although the data supporting trends in the genetic patterns along the longitudinal gradient of Kettle Creek are not conclusive, the patterns are worthy of discussion. These patterns could be explained by the presence of a continuum of overlapping bacterial plumes. If patches of different genetic types exist along the stream and continue to release clones into the water column, we would expect to find much more homogeneity at the headwaters than farther downstream. Water flowing through downstream sites would carry a composite of upstream genetic types and therefore would yield an assemblage of bacteria with high degrees of genetic variation. The hypothesis presented by McArthur et al. (25), that genetic diversity is positively correlated with habitat variability, may explain the increase in genetic variation of the *J. lividum* population along the course of Kettle Creek. Several studies have proposed and demonstrated an increase in habitat variability from the headwaters to medium-sized reaches (7, 42). A high degree of diversity of dissolved organics in the lower reaches of Kettle Creek may explain the apparent, high degree of genetic variation in the *J. lividum* groups found in the same reaches.

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