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Genetics

Genetic transfer in *Desulfovibrio desulfuricans*

(bacteriophage/genetic recombination/sulfate-reducing bacteria/transduction)

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ABSTRACT **An apparently defective bacteriophage capable of mediating transduction has been identified in culture filtrates of *Desulfovibrio desulfuricans* (American Type Culture Collection 27774). Phage-mediated intraspecies transfer of antibiotic resistance markers occurs with a frequency of 10^{-5} to 10^{-6} per recipient cell. The vector contains linear fragments of double-stranded DNA of about 13.5 kilobase pairs, which appear to be random pieces of bacterial DNA. As yet, neither induction nor plaque formation has been observed. To our knowledge, a system of genetic exchange has not been described before for a member of the sulfate-reducing bacteria.**

The sulfate-reducing bacteria are a heterogeneous group of strictly anaerobic Gram-negative microorganisms that are capable of dissimilatory sulfate reduction. These organisms play critical roles in the global sulfur cycle, microbial metal corrosion, and anaerobic digestors (1). However, detailed studies of the physiology of these bacteria have been hampered by difficulties with standard microbiological manipulations, particularly colony formation. Only recently has their metabolic versatility begun to be appreciated (2, 3). With the advent of molecular biological tools, the metabolic processes of these recalcitrant organisms are beginning to be accessible. For example, the hydrogenase genes from *Desulfovibrio vulgaris* (Hildenborough) have been cloned (4, 5), and some *D. vulgaris* promoters have been shown to function in *Escherichia coli* (6, 7). As problems with the manipulations of these bacteria are overcome, mutants are now being isolated as well (8). A genetic exchange system has not been reported, but would greatly increase the analytical potential of a mutational approach to the metabolism of the sulfate reducers. Handley *et al.* (9) reported the observation of bacteriophage-like particles in a mitomycin C-treated culture of *D. vulgaris*; however, no further characterization was given. We report here a defective bacteriophage from *Desulfovibrio desulfuricans* American Type Culture Collection (ATCC) 27774, named Dd1, apparently capable of generalized transduction.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *D. desulfuricans* ATCC 27774 was kindly provided by H. Peck (University of Georgia, Athens). Strains MO301, MO302, MO303, and MO305 were selected as spontaneous mutants of strain 27774 resistant to 100 μg of rifampicin per ml, 200 μg of nalidixic acid per ml, 150 μg of neomycin sulfate per ml, and 150 μg of novobiocin per ml, respectively.

The growth medium, lactate-sulfate (LS), was adapted from ref. 8 and was composed of 20 mM NH_4Cl, 2.2 mM KH_2PO_4, 8 mM $MgSO_4 \cdot 7H_2O$, 0.6 mM $CaCl_2 \cdot 2H_2O$, 50 mM Na_2SO_4, 60 mM sodium DL-lactate (60% wt/vol syrup, Sigma), 25 mM Hepes (pH 7.0), 12.5 ml of mineral solution per liter (10), 1.0 ml of 10× vitamin solution per liter (10), and 0.1% yeast extract (Difco). Resazurin (2.5 ml of a 0.1% stock solution per liter of medium) was used to indicate the reductive state. The medium, in a round-bottom flask in a heating mantle, was continuously flushed with argon while it was brought to a boil. After boiling, the medium was removed from the heat, and 40 ml of reducing agent containing 200 mM NaOH, 160 mM DL-cysteine, and 100 mM $Na_2S \cdot 9H_2O$ was added per liter. The pH of the medium was then adjusted to 6.8–7.0 with 3 M HCl. Before autoclaving, the flasks were sealed with rubber stoppers wired in place. For solidified medium, 16.2 g of Bacto-agar (Difco) and 20 mg of $FeCl_2 \cdot 4H_2O$ were added per liter. The soft agar used for overlayers contained 20 mM Hepes (pH 7.0) and 5 mg of $FeCl_2 \cdot 4H_2O$, 2.5 ml of resazurin solution, 40 ml of reducing agent, and 11 g of Bacto-agar per liter.

Anaerobic growth was achieved in liquid culture in Hungate tubes sealed with black rubber stoppers and was measured as an increase in optical density at 660 nm using a Coleman Junior II spectrophotometer. Subculturing, plating, and transduction assays were performed in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) filled with a nitrogen/hydrogen atmosphere (*ca.* 95:5 percentage composition) and kept at 30°C.

Isolation of Bacteriophages. Bacteriophages were isolated from LS cultures of the antibiotic-resistant strains at mid- to late-exponential phase. For a large phage preparation, a 500-ml culture was centrifuged at 16,000 × *g* for 15 min at 4°C, and the supernatant was passed through a 0.22-μm pore-sized cellulose acetate filter. The phage were concentrated by a second centrifugation at 108,000 × *g* for 1 hr at 24°C. The phage pellet was resuspended in a minimal amount (usually 0.5 ml) of fresh LS medium. Smaller amounts of phage were concentrated by using a TLA100.3 rotor at 108,000 × *g* for 1 hr at 24°C in a TL100 Beckman tabletop ultracentrifuge.

Transduction Assay. The wild-type strain 27774, grown to midexponential phase, [≈8×10^8 colony-forming units (cfu)/ml] was used as the recipient. Cell-free filtrates (0.2-μm pore-sized sterile cellulose acetate filters) of the antibiotic resistant strains were the phage source. Typically, 0.5-ml aliquots of the recipient culture were incubated with 0.5-ml volumes of donor filtrate for ≈12–18 hr at 30°C. Samples of 100 and 200 μl of the mixture were added to molten soft agar, which was layered onto solidified medium. The appropriate antibiotic [100 μg of rifampicin per ml, 150 μg of neomycin sulfate per ml, 200 μg of nalidixic acid per ml, or 150 μg of novobiocin per ml (final plate concentration)] was added to the soft agar and plated along with the assay sample. A 100-μl aliquot of the donor filtrate was plated to test for colony-forming units remaining in the filtrate. For an estimation of the spontaneous resistance occurring in the recipient strain, a 100-μl cell sample was plated with the appropriate antibiotic. Plates were incubated in the anaerobic chamber at 30°C. Generally, after a week, transductants could be counted. The frequency of antibiotic transfer was calculated as the ratio of

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the number of transductants to the colony-forming units of the recipient cells in the incubation mixture. The recipient colony-forming units were determined at the beginning of the transduction assay by dilution and plating in soft agar overlayers.

DNA Extraction and Restriction Analysis. For DNA preparation, the phage pellet obtained from a large culture was resuspended in 0.5 ml of TE buffer (10 mM Tris·HCl/1 mM EDTA, pH 8.0) per liter of the original culture volume. The DNA was extracted twice with an equal volume of 1:1 (vol/vol) chloroform/phenol [the chloroform contained isoamyl alcohol, 24:1 (vol/vol), and redistilled phenol contained 0.1% hydroxyquinoline and was equilibrated with 0.1 M Tris (pH 8.0)]. Next, the DNA was precipitated with 0.1 vol of 3 M sodium acetate and 2 volumes of absolute ethanol at −20°C and was centrifuged at 18,000 × *g* for 20 min. The DNA precipitate was dissolved in 50 μl of TE buffer, and DNA was stored at 4°C. Enzyme digests of the phage DNA were performed at 37°C for 1 hr under the conditions recommended by the suppliers [*Eco*RI and RNase-free DNase I, Promega Biotec (Madison, WI); *Hin*dIII, Bethesda Research Laboratories]. Native phage DNA, enzyme digests, and molecular weight markers (phage λ *Hin*dIII digest, Bethesda Research Laboratories) were subjected to electrophoresis in a horizontal 0.5% agarose gel (standard low molecular weight agarose; Bio-Rad) in buffer composed of 89 mM Tris, 89 mM boric acid, and 2 mM EDTA (pH 8.0) at 100 V (constant voltage) for 7 hr at room temperature.

Genomic DNA was prepared from *D. desulfuricans* strain 27774 by the procedure of Marmur (11). Precipitation, digestion, and electrophoresis of the bacterial DNA were performed as described for the phage DNA.

Electron Microscopy. The phage pellet obtained from ultracentrifugation was gently resuspended in about 50 μl of 100 mM ammonium acetate. This suspension was mixed with an equal volume of 2% (wt/vol) phosphotungstic acid (brought to pH 7.2 with KOH), and then 2 μl was placed on carbon-coated grids. After air-drying, the grids were examined with a JEOL (Peabody, MA) 100B electron microscope.

RESULTS

Attempts to identify a genetic exchange system for the sulfate-reducing bacteria have led to the discovery of an apparently generalized transducing agent. When a rifampicin-resistant derivative of *D. desulfuricans* strain 27774 was mixed with a nalidixic acid-resistant derivative of that same strain and incubated overnight, the number of colony-forming units obtained from the mixture that were simultaneously resistant to rifampicin and nalidixic acid was significantly increased over the level of spontaneous mutation in pure cultures. To examine the mechanism of the presumptive genetic recombination, cell-free filtrates of the drug-resistant mutants were prepared and added to the drug-sensitive wild-type cells. In every case tested, antibiotic-resistant derivatives were obtained in excess over the spontaneous appearance of resistant colonies (Table 1); therefore, gene transfer was mediated by a filterable agent. Because DNase (1 mg/ml) added to the incubation mix did not affect the transfer frequency (data not shown), the presence of a transducing particle was inferred.

The frequencies of transfer of resistance to nalidixic acid, neomycin sulfate, novobiocin, or rifampicin were in the range of 10^{-5} to 10^{-6} per recipient colony-forming unit. The transferred resistance markers were stable in that subcultured transductants retained the appropriate drug resistance under nonselective conditions for at least 13 generations. In addition, a neomycin-resistant transductant was able to donate neomycin resistance to the wild-type parent at a frequency similar to that given by the original donor.

Table 1. Gene transfer activity of *D. desulfuricans* ATCC 27774 derivatives

Donor	Donor phenotype*	Antibiotic-resistant cfu†	
		− filtrate	+ filtrate
MO301	Rif^R	24	1715
MO302	Nal^R	10	8090
MO303	Neo^R	19	2030
MO305	Nov^R	0	6616

*Rif^R, resistant to rifampicin; Nal^R, resistant to nalidixic acid; Neo^R, resistant to neomycin sulfate; Nov^R, resistant to novobiocin.
†cfu per ml of recipient cells in assay mixture. The recipient was wild-type *D. desulfuricans* strain 27774 at 5×10^8 to 1.5×10^9 cfu/ml.

These results suggested that transduction might be the mechanism of genetic exchange. To date, however, no plaque-forming ability has been observed with cell-free filtrates of derivatives of this *D. desulfuricans* strain. No increase in gene-transfer frequency was seen in filtrates after mitomycin C treatment of donor cultures [5 μg/ml for 5 min (12)], nor did lysis of the treated culture occur. Inspection of the material obtained in pellets from high-speed centrifugation of the cell-free culture filtrates revealed a uniform population of bacteriophage-like particles (Fig. 1). Controls confirmed that gene transfer ability was removed from the filtrate with centrifugation. Fig. 1*a* contains a cluster of particles among bacterial flagella that pelleted in some preparations. Individual phage particles in Fig. 1*b* exhibit the structure of the head and the short tail, which has no visible sheath or appendages. This type of phage particle is typical of those belonging to Bradley group C (13). The head is approximately 43 nm in diameter, and the tail measures 7.1 nm in length and 5.7 nm in width. Three separate phage preparations were viewed with the same basic structure and size observed. This bacteriophage particle has been named Dd1.

To determine the nature of the nucleic acid contained in the phage heads, a larger phage preparation was extracted, and the nucleic acid was visualized in agarose gels following electrophoresis and staining with ethidium bromide (Fig. 2). Because of its sensitivity to degradation by RNase-free DNase I (Fig. 2, lane c) and to digestion by the endonucleases *Eco*RI (Fig. 2, lane d) and *Hin*dIII (not shown), the nucleic acid from Dd1 is most likely double-stranded DNA. This result is consistent with the phages of Bradley group C

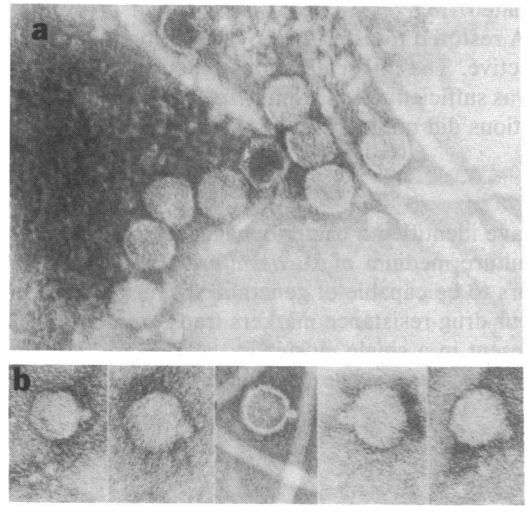

FIG. 1. Electron micrographs of a cluster of phage particles (*a*) and individual phage particles (*b*) from MO302. The phage were prepared as described. (×157,500.)

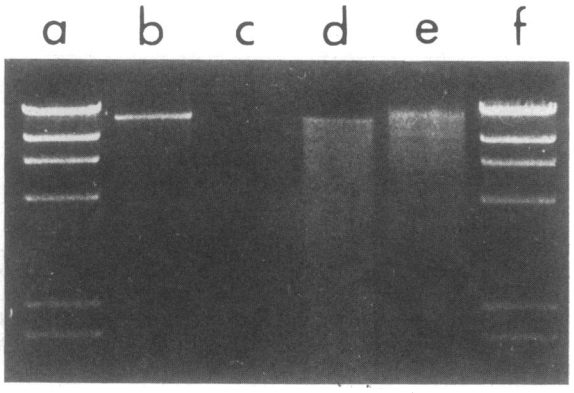

FIG. 2. Electrophoretic migration of phage λ DNA cut with *Hin*dIII (lanes a and f), uncut Dd1 DNA (lane b), RNase-free DNase I-treated Dd1 DNA (lane c), *Eco*RI digest of Dd1 DNA (lane d), and *Eco*RI digest of MO302 genomic DNA (lane e).

morphology, which generally contain double-stranded DNA (13). The DNA extracted from Dd1 appeared to be of a uniform size; length estimates from three different preparations averaged 13,600 base pairs (bp) (Fig. 2, lane b). The restriction endonuclease digests of Dd1 DNA gave a reasonably uniform distribution of smaller fragment sizes, resulting in a smear in agarose gels (Fig. 2, lane d). There was no evidence for multiple copies of phage-specific sequences being preferentially packaged in phage heads. An *Eco*RI digestion pattern of *D. desulfuricans* mutant MO302 genomic DNA also did not show any highly repetitive sequences (Fig. 2, lane e). Although all genetic markers tested have been transferred to date, further experiments are needed to determine whether the DNA in Dd1 represents a random packaging of bacterial sequences (14).

In preliminary experiments to examine the requirements for the transduction process, the time of incubation of Dd1 and recipient cells, stability of Dd1 in filtrates, and cation requirements were examined. The minimum incubation time of the transduction mixture for optimum transfer frequency was found to be 2–4 hr with no significant change in the number of transductants up to about 12 hr. The phage filtrate was quite stable at 30°C, with no apparent change in transduction frequency over 24 hr and only a 30% decrease after 72 hr. When EDTA was added to the transduction mixture in excess relative to the divalent cations, genetic transfer was eliminated. Mg^{2+}, Mn^{2+}, or Ca^{2+} added in excess of the EDTA restored transfer; monovalent cations generally were ineffective. The divalent cation content of the transduction mix was sufficient for maximum transduction, as supplemental cations did not increase the frequency.

DISCUSSION

We have identified a bacteriophage-like particle, Dd1, from the culture medium of *D. desulfuricans* ATCC 27774 that appears to be capable of generalized transduction. Because the four drug-resistance markers transferred are unlikely to be present in a single cluster in the *D. desulfuricans* chromosome, it is possible that any selectable gene can be mobilized in this vector. Differences in the frequencies of transfer of the resistances (Table 1) were seen consistently in several experiments and could be due to marker effects, to the efficiency of recombination and expression of the drug resistance, or to the efficiency of packaging the genes into phage particles. Routinely, frequencies of 10^{-5} to 10^{-6} were obtained in transduction experiments. This may be improved as parameters affecting the process are identified and optimized.

The morphological characteristics of the Dd1 phage particles resemble those of T7 or T3 coliphages, except that these phages are slightly larger (63-nm head diameter vs. 43-nm for Dd1) and concomitantly package more DNA (*ca.* 40 kbp vs. 13.5 kbp, respectively) (15). The nucleic acid extracted from the Dd1 heads was quite uniform in length but appeared to be a collection of random fragments when digested with restriction endonucleases, a result that supports the extrapolation that any marker may be transduced.

Characteristic bacteriophage properties, such as plaque formation and induction, have not been observed. Although tests to obtain an indicator strain should be continued, the defective nature of this particle is analogous to that described for a generalized transducing agent of *Rhodobacter capsulatus* (16), which has no preferential packaging of phage-encoding DNA sequences and is detected only by gene transfer (14).

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