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Recovery of temperate *Desulfovibrio vulgaris* bacteriophage using a novel host strain

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Summary

A novel sulfate-reducing bacterium (strain DePue) closely related to *Desulfovibrio vulgaris* ssp. *vulgaris* strain Hildenborough was isolated from the sediment of a heavy-metal impacted lake using established techniques. Although few physiological differences between strains DePue and Hildenborough were observed, pulse-field gel electrophoresis (PFGE) revealed a significant genome reduction in strain DePue. Comparative whole-genome microarray and polymerase chain reaction analyses demonstrated that the absence of genes annotated in the Hildenborough genome as phage or phage-related contributed to the significant genome reduction in strain DePue. Two morphotypically distinct temperate bacteriophage from strain Hildenborough were recovered using strain DePue as a host for plaque isolation.

Introduction

Viruses are thought to be among the most abundant biological entities on earth (Chibani-Chennoufi *et al.*, 2004). Lytic viruses have long been implicated in the biogeochemical cycling of nutrients (Fuhrman, 1999) and, more recently, their role in shaping both the structure and evolution of microbial systems has received increased attention (Jiang and Paul, 1998; Weinbauer and Rassoulzadegan, 2004). For example, viral-mediated lateral gene transfer has been suggested to contribute to genome variation observed among strains of the same species (Lindell *et al.*, 2004). Although viral-mediated processes are of increasingly recognized importance, little is known about their activities in natural systems encompassing both oxic and anoxic habitats. In particular, very little is known about the activity of viruses in anoxic systems populated by obligate anaerobes.

Sulfate-reducing bacteria (SRB) are among the better characterized of anaerobes, constituting a physiologically and phylogenetically diverse assemblage that are abundant in both marine and terrestrial habitats. SRB have considerable environmental and applied significance (Devereux *et al.*, 1989; Widdel and Bak, 1992; Rabus *et al.*, 1999–2005), serving a central role in the biogeochemical cycling of carbon and sulfur species. They have been employed for the bioremediation of metals and toxic compounds (Sun *et al.*, 2000; Davis *et al.*, 2002; Payne *et al.*, 2002), but are also implicated in biocorrosion (Dinh *et al.*, 2004). *Desulfovibrio vulgaris* has served as an important model organism for this functional assemblage, having a well-developed genetic system that is now complemented by the availability of a complete genome sequence.

The genome sequence of *D. vulgaris* Hildenborough suggests the presence of two nearly identical mu-like bacteriophages, one lambdoid bacteriophage and the remnants of a fourth bacteriophage genome (Heidelberg *et al.*, 2004). Genes coding for tail-fibre proteins, Cro/cI-transcriptional regulators and phage-integrase proteins suggest the presence of two other undefined bacteriophage within *D. vulgaris* Hildenborough. This annotation is consistent with earlier studies that demonstrated the induction of phage-like particles using mitomycin C or UV radiation (Handley *et al.*, 1973; Seyedirashti *et al.*, 1991). Although molecular characterization demonstrated that these particles were comprised of at least two distinct viruses (Seyedirashti *et al.*, 1992), the lack of a suitable indicator strain prevented further studies of *Desulfovibrio* bacteriophage biology. Here, we report the isolation and partial characterization of a novel strain of *D. vulgaris* capable of serving as a host for the isolation of two morphotypically distinct temperate bacteriophages from *D. vulgaris* ssp. *vulgaris* strain Hildenborough.

Results

Strain DePue was isolated from a backwater lake on the Illinois River. The lake sediments are heavily contami-

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nated with heavy metals from the historical operation of a zinc smelting plant sited on the lake (Webb *et al*., 2000). Combined activity and molecular analyses indicated that SRB comprised a significant fraction of the microbial population in the more highly contaminated sediments (D.A. Stahl, unpubl. data). Along with this culture-independent analysis, we isolated nine *Desulfovibrio*-like bacteria from Lake DePue (Illinois) using the freshwater medium of Widdel and Bak (1992) amended with sulfate and lactate. Sequence analysis of most of the 16S rRNA gene, the complete 16S-23S internal transcribed spacer (ITS) region, a small portion of the 23S rRNA gene, as well as most of the A and B subunits of the dissimilatory sulfite reductase (*dsrAB*) gene revealed that all nine isolates were identical in these regions of the genome, and therefore likely derived clonally from the same strain (designated strain DePue).

Desulfovibrio vulgaris Hildenborough and strain DePue are related at greater than 99% 16S rRNA [four transitions at base pairs (bp) 110, 142, 1570 and 1850, one deletion at bp 1826] and *dsrAB* gene (one transition at bp 1322) sequence similarity. Initial physiological comparisons revealed little or no variation in growth rate or substrate utilization (Tables 1 and 2). Both strains oxidize lactate incompletely to acetate and utilize sulfate, sulfite, thiosulfate and fumarate as electron acceptors. Fermentative growth was observed using pyruvate and malate.

Pulse-field gel electrophoresis (PFGE; Fig. 1) analysis of I-CeuI or NotI restriction endonuclease digestion of the DePue genomic DNA revealed a major genome reduction in strain DePue (approximately 70 000 kb). In order to better resolve the genomic differences between the strains, a whole-genome microarray based on the *D. vulgaris* ssp. *vulgaris* strain Hildenborough sequence was used to analyse DNA from strain DePue. This revealed that approximately 90% of the open reading frames (ORFs) present in strain Hildenborough had orthologues in stain DePue. It also identified six distinct

Table 1. Maximum growth rate (h^{-1}) of *Desulfovibrio vulgaris* strains at varying temperatures.

Strain	20°C	25°C	30°C	37°C	40°C	45°C
Hildenborough	0.038	0.084	0.11	0.13	0.16	0.14
DePue	0.037	0.087	0.11	0.12	0.15	0.13
DePue_2	0.037	0.087	0.11	0.11	0.16	0.15
PT2	0.036	0.067	0.095	0.12	0.19	0.06

Table 2. Electron donors and acceptors utilized by strain DePue.

	None	$SO_4^=$	$SO_3^=$	$S_2O_3^=$	DMSO	Fumarate	Fe(III)	NO_3^-
H_2	–	–	–	–	–	–	–	–
H_2/Ac	–	+	+	+	–	–	–	–
Acetate	–	–	–	–	–	–	–	–
Propionate	–	–	–	–	–	–	–	–
Ethanol	–	+	+	+	–	–	–	–
Lactate	–	++	++	++	–	+	–	–
Isopropanol	–	–	ND	ND	ND	ND	ND	ND
2-Propanol	–	–	ND	ND	ND	ND	ND	ND
1,2-Propanediol	–	–	ND	ND	ND	ND	ND	ND
1-Butanol	–	–	ND	ND	ND	ND	ND	ND
2-Butanol	–	–	ND	ND	ND	ND	ND	ND
Pyruvate	+	+	ND	ND	ND	ND	ND	ND
Butyrate	–	–	ND	ND	ND	ND	ND	ND
Succinate	–	–	ND	ND	ND	ND	ND	ND
Fumarate	–	–	ND	ND	ND	ND	ND	ND
Malate	(+)	(+)	ND	ND	ND	ND	ND	ND
Fructose	–	–	ND	ND	ND	ND	ND	ND
Glucose	–	–	ND	ND	ND	ND	ND	ND
Methanol	–	–	ND	ND	ND	ND	ND	ND
Glycerol	–	–	ND	ND	ND	ND	ND	ND
Glycine	–	(+)	ND	ND	ND	ND	ND	ND
Alanine	–	–	ND	ND	ND	ND	ND	ND
Choline	–	–	ND	ND	ND	ND	ND	ND
Valerate	–	–	ND	ND	ND	ND	ND	ND
Isovalerate	–	–	ND	ND	ND	ND	ND	ND
Furfural	–	–	ND	ND	ND	ND	ND	ND
Formate	–	–	ND	ND	ND	ND	ND	ND

++, excellent growth; +, good growth; (+), poor growth; –, no growth; ND, not determined.

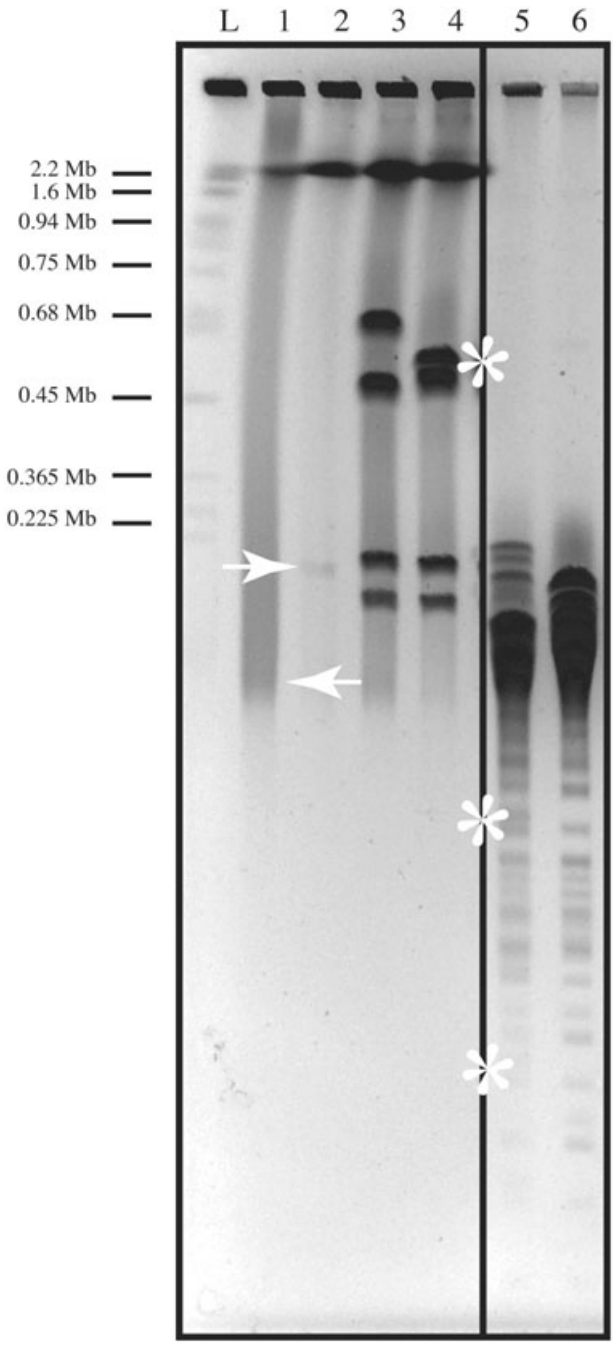

Fig. 1. Pulse-field gel electropherogram of *Desulfovibrio vulgaris* strains Hildenborough and DePue. Stars highlight differences between the two strains and arrows indicate extrachromosomal (plasmid) DNA. Lane L – size marker (*Saccharomyces cerevisiae*); lane 1 – *D. vulgaris* Hildenborough genomic DNA; lane 2 – strain DePue genomic DNA; lane 3 – strain Hildenborough DNA digested with I-CeuI; lane 4 – strain DePue DNA digested with I-CeuI; lane 5 – strain Hildenborough DNA digested with NotI; lane 6 – strain DePue DNA digested with NotI.

regions, containing a total of 295 genes, that are apparently absent in the genome of strain DePue (Table 3 and Fig. 2 , see Table S1 in *Supplementary material* for a complete list of genes). While the majority (188) of the missing genes are annotated as conserved hypothetical or hypothetical proteins, 55 are annotated as phage and phage-related proteins. With the exception of a putative tail-fibre assembly protein (DVU0221), virion morphogenesis protein (DVU1119) and four hypothetical proteins (one of which lies within a known phage operon; DVU1733, DVU1784, DVU1751, DVU2847), all the genes annotated in the Hildenborough genome as phage or phage-related are absent in strain DePue.

Comparison of PFGE and microarray data indicated that strains DePue and Hildenborough share the same complement of genes in the 133 and 434 kb restriction fragments common to both, corresponding to bp 2 560 462–2 693 868 and bp 3 248 263–109 784 in strain Hildenborough. The digestion product corresponding to the 554 kb fragment in Hildenborough (bp 2 693 869–3248 262) is reduced in strain DePue by approximately 75 kb, consistent with the absence of hybridization of DePue DNA to probes encompassing a similar amount of genomic material within this region of the Hildenborough genome. Although deletion of the phage-related genes from the 2259 kb digestion product (bp 298 781–2 560 461 in strain Hildenborough) should reduce the genome size of strain DePue by approximately 127 kb, gel resolution was not sufficient to resolve this size variation.

Restriction digestion and microarray hybridization comparison suggested that strain DePue also contains unique sequences. Although a deletion of 37 kb from the 184 kb I-CeuI digestion product of strain Hildenborough (bp 109 785–298 780) should be readily apparent by PFGE, this was not observed. Fragments of near identical size in both strains suggested that DePue contains DNA in this region absent in the corresponding Hildenborough fragment. This was confirmed by a limited polymerase chain reaction (PCR)-based survey of correspondence between gene order and sequence similarity between the two strains. Primers were designed to flank regions of phage-gene deletion, and tested using DNA from strains Hildenborough and/or DePue. Polymerase chain reaction amplicons spanning three phage gene-deleted regions in strain DePue were recovered using primers targeting flanking sequences in strain Hildenborough. These correspond to regions R2–R4 of the 2259 kb I-CeuI fragment of strain Hildenborough. Polymerase chain reaction amplicons spanning the phage gene-deleted regions R1, R5 and R6 could not be recovered from strain DePue, nor could PCR amplicons encoding for phage-specific genes location within these regions. Sequence analysis of the three DePue amplicons confirmed the absence of phage-like ORFs and revealed segments of high nucleotide sim-

Table 3. Distribution of genes present in the Hildenborough genome, but absent in the genome of strain DePue.

Region	Length (kb)	CHP/HP ORFs	Phage-related ORFs		Other ORFs
			Tail/tail-fibre-related	Other	
R1	36.7	26	3	4	11
R2	36.0	24	3	1	10
R3	39.4	50	1	2	12
R4	51.9	29	7	7	3
R5	32.1	31	9	4	10
R6	40.7	28	10	4	6
Total	236.8	188	33	22	52

CHP, conserved hypothetical protein; HP, hypothetical protein.

ilarity (98–99%) for both coding and non-coding regions flanking the regions lacking phage-like genes (Fig. 3). Our inability to recover amplicons from strain DePue using several different sets of primers targeting sequences flanking the other phage-gene locals suggested sequence rearrangement or divergence in these regions.

DNA sequences flanking Region 4 were virtually identical for both strains (Fig. 3). Direct repeats of 51 nucleotides were encountered immediately upstream and downstream of this phage-encoding region in strain Hildenborough. The upstream direct repeat resides entirely in an arg-tRNA. The DePue strain contains a single copy of this sequence in the same tRNA gene. Similarly, direct repeats of 17 nucleotides immediately flank both ends of phage-encoding Region 3 for strain Hildenborough. Again, one of the repeats is located within a tRNA encoding ORF, although this tRNA (serine anticodon) is on the opposite strand of the upstream region. The DePue strain contains only one copy of this sequence, located in a serine tRNA. Region 3 flanking DNA also contained two short segments (63 bp and 180 bp) of greater sequence divergence, sharing less than 50% nucleotide similarity between strains (Fig. 3).

The greatest sequence divergence between strains was observed for DNA downstream of Region 2 (Fig. 3), including apparent insertion or deletion. This downstream segment contained sequences unique to strain DePue and genomic rearrangement of a 785 bp fragment (designated A in Fig. 3). This fragment, found in the Hildenborough genome at bp 1 245 835–1 246 618 (approximately 4.5 kb downstream), is entirely located within an ORF annotated as a hypothetical protein.

The genome segments flanking putative phage-encoding Region 2 contained two other unique features. Sequence analysis indicated the ORF immediately downstream of the putative phage-encoding region (DVU1145, annotated as a hypothetical protein in strain Hildenborough) was absent from this location in strain DePue. However, comparative microarray analysis gave a positive result for this gene, suggesting its presence elsewhere in the genome of strain DePue. No direct repeats flanked the missing phage-encoding region in strain DePue; however, there is a 53 bp fragment unique to strain DePue at this location.

The absence of common lysogenic phage and near identity to strain Hildenborough suggested that strain DePue might serve as a host for the temperate bacteriophage previously shown to be induced in strain Hildenborough (Handley *et al*., 1973; Seyedirashti *et al*., 1991; 1992). These phages could not be propagated on other *Desulfovibrio* or enteric bacteria (*Escherichia coli* or *Salmonella typhimurium*) as they failed to form plaques on these strains (Seyedirashti *et al*., 1991). Using a standard plaque assay, we recovered infectious particles from plaques formed upon bacterial lawns of strain DePue. Scanning electron microscopic examination revealed two distinct icosohedral bacteriophages differing in tail structure (bent or straight) and head size (Fig. 4). The bent-tailed phage had a head diameter of 100 nm versus 50 nm

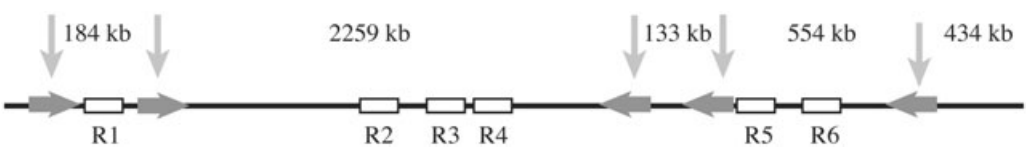

Fig. 2. *Desulfovibrio vulgaris* Hildenborough chromosome map highlighting the putative phage-encoding regions (white boxes) absent in the genome of strain DePue. The map begins at *oriR*. The vertical arrows show the I-CeuI restriction site locations. Approximate fragment sizes are listed above each fragment. The horizontal arrows indicate both the location and direction of the five rRNA operons.

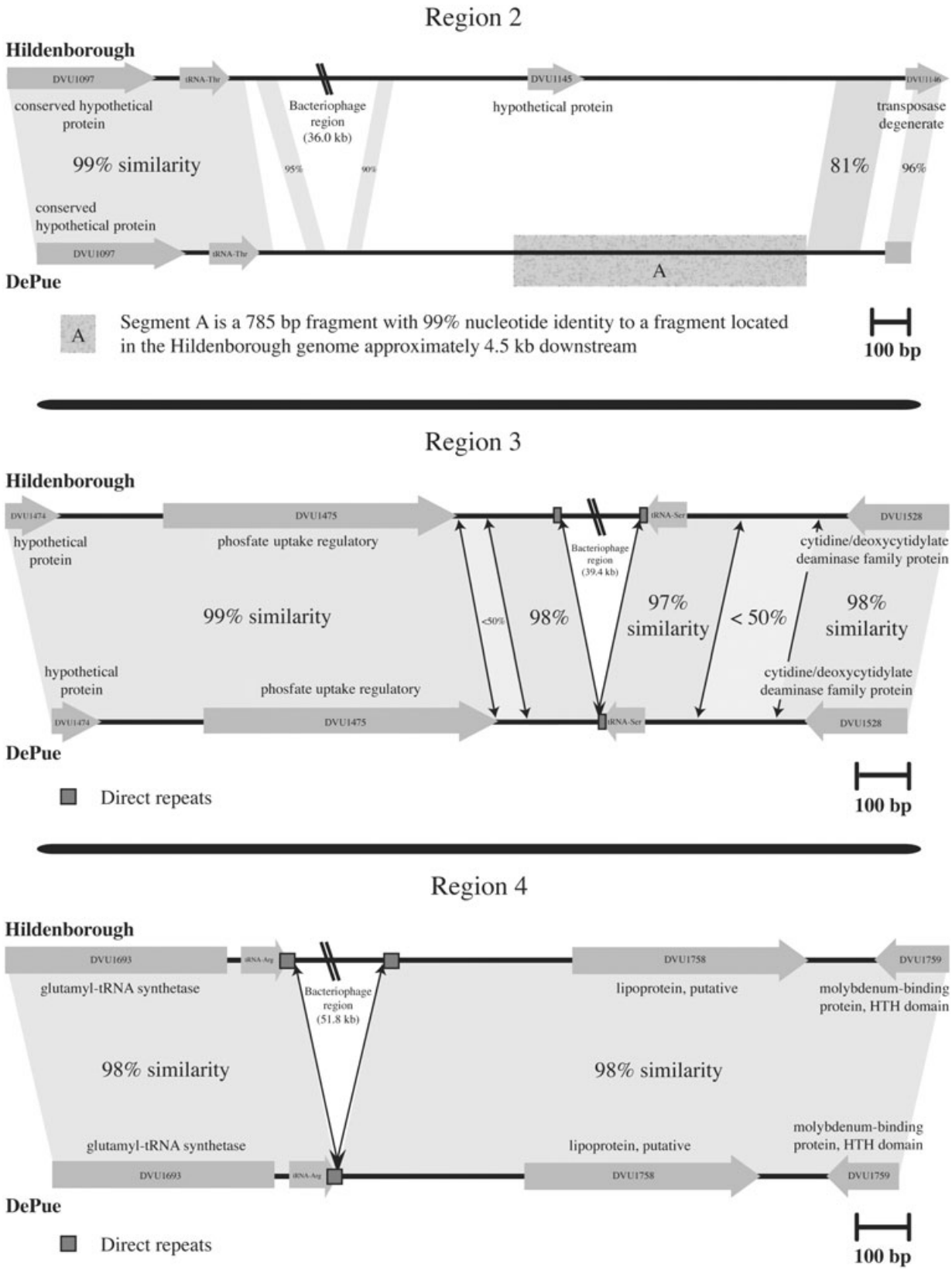

Fig. 3. Comparison of Regions 2, 3 and 4 with the putative phage-encoding sites and associated flanking regions for strains Hildenborough and DePue. HTH, helix–turn–helix.

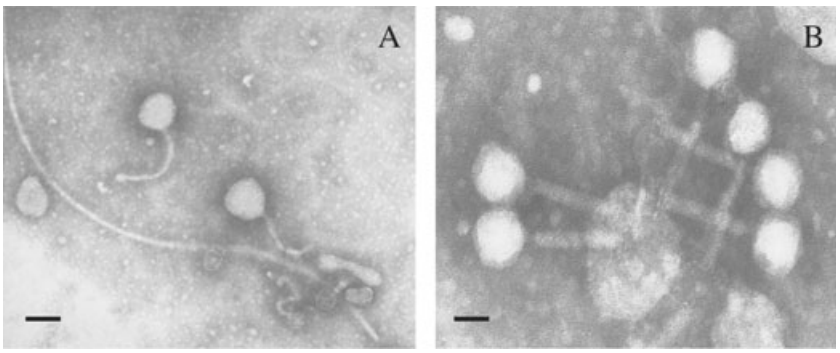

Fig. 4. Scanning electron micrographs of bacteriophage particles isolated from *Desulfovibrio vulgaris* Hildenborough. Scale bar represents 100 nm in (A) and 50 nm in (B).

for the straight-tailed isolate. In comparison, the bacteriophage induced by Handley and colleagues (1973) were all reported to have heads measuring approximately 50 nm in length and width. Thus, the bent-tailed bacteriophage were most likely not recovered in the earlier study.

Infection of strain DePue by bacteriophage particles originating from strain Hildenborough was confirmed by the isolation of a lysogenic variant of strain DePue recovered from one of the plaques. Polymerase chain reaction amplicons specific for bacteriophage genes present in the R2 region of the Hildenborough genome were recovered from DNA isolated from the lysogenized strain of DePue. Sequence analysis confirmed these to be virtually identical to corresponding amplicons generated from strain Hildenborough (minor sequence variation is attributable to *Taq* polymerase error). No amplicons could be generated from the original strain DePue. Thus, plaques on DePue lawns could be directly linked with lysogenic bacteriophage previously identified in strain Hildenborough.

Discussion

Sulfate-reducing bacteria have been isolated from a variety of contaminated environments with several capable of reducing and precipitating heavy metals. *Desulfovibrio* species in particular have been shown to reduce a variety of oxidized metals, including Fe(III), Cr(VI), U(VI), Mn(IV), As(V) and Tc(VII) (Lovley and Phillips, 1992; 1994; Lovley *et al*., 1993; Lloyd *et al*., 2000; Macy *et al*., 2000). Thus, heavy-metal impacted anoxic systems, as represented by the sediments of Lake DePue, may be enriched in SRB such as *Desulfovibrio*. Given the recent reports describing genomic size variation between environmental isolates of the same genus (Rocap *et al*., 2003) and species (Thompson *et al*., 2005), genome size variation among *D. vulgaris* isolates is not unprecedented. Unlike the previously described genomic and allelic variations among environmental isolates, the reduction in genome size of strain DePue is directly correlated with the absence of genes encoding for phage and phage-related elements. However, we cannot as yet eliminate the possible presence of lysogenic phage elements unique to strain DePue.

The isolated bacteriophage reported here might serve to complement the existing suite of tools used for shuttling genetic material into *Desulfovibrio* species (Voordouw, 1995). Only two methods currently exist for genetic manipulation of *D. vulgaris*: an actively transposing insertion sequence (Fu and Voordouw, 1998) and a modified mini-Tn*5* transposon (J. Wall, pers. comm.).

Without knowing the evolutionary history of strains Hildenborough and DePue, we cannot resolve the contribution of gain or loss to the observed distribution of lysogenic bacteriophage. Each of the three characterized putative phage-excision regions in strain DePue is located immediately upstream or downstream of a tRNA gene. In the genome of strain Hildenborough, the remaining three uncharacterized putative phage-excision regions are also located immediately upstream or downstream of sequence motifs characteristic of bacteriophage insertion sites (Campbell, 1992; Hacker and Kaper, 2000; Williams, 2002; Campbell, 2003). Our inability to generate PCR amplicons bridging three of the phage-encoding regions in strain DePue also points to significant sequence divergence that must be evaluated using an alternative sequencing strategy.

These observations raise the immediate question of the possible contribution lysogenic phages play in *Desulfovibrio* fitness. The role of phage-encoded genes to pathogen virulence is well documented (Boyd and Brussow, 2002). Although phage contributions to host fitness in open environmental systems are not well explored, introduction of genes embedded within bacteriophage genomes might facilitate microbial growth in nutrient-stressed systems (Miller *et al*., 2003). This is supported by the recent discovery of photosynthetic genes in temperate *Prochlorococcus* phage (Lindell *et al*., 2004), suggestive of a direct contribution to host fitness. In contrast, experiments performed with *E. coli* suggested no significant cost or benefit to carrying phage DNA (Bohannan

and Lenski, 2000) and we have yet to discern gross physiological differences between strains DePue and Hildenborough, nor have we found any genes for improved fitness within the bacteriophage regions.

At this time we can only speculate that the environment from which strain DePue was isolated, the heavy-metal impacted sediments of Lake DePue, may have contributed to excision of endogenous phage elements. Stress contributing to DNA damage is well recognized to induce excision and lytic propagation of lysogenic phage. We anticipate that completed and ongoing genome sequencing may provide additional perspectives on the presence and distribution of phage among sulfate-reducing microorganisms and their relatives. Genome sequences are now available for two relatives of *Desulfovibrio*, the Deltaproteobacteria *Geobacter sulfurreducens* PCA (Methe *et al*., 2003) and *Desulfotalea psychrophila* Lsv54 (Rabus *et al*., 2004). Neither the annotation of these two genomes nor that of another sulfate-reducing prokaryote, *Archaeoglobus fulgidus* DSM 4304 (Klenk *et al*., 1997), report the presence of prophage, although several phage-related genes are present within the genome of *D. psychrophila*. The absence of prophage might reflect the very limited sampling of organisms and habitats (Ramirez and Tomasz, 1999; Brussow *et al*., 2004). Certainly, the completion of nine SRB and 19 other Deltaproteobacteria genomes now in progress (Bernal *et al*., 2001) will provide a better sampling.

We anticipate that the availability of this novel *D. vulgaris* strain could provide a useful model system for exploring the role of temperate phage in the life history of this species and related SRB. For example, the lysogenized variants of strain DePue might provide for direct evaluation of the influence of phage integration on host physiology, stress response and niche adaptation. The availability of phage might also serve to expand the repertoire of genetic tools for *D. vulgaris* and other *Desulfovibrio* species.

Experimental procedures

Isolation of strains

Sediment from Lake DePue (Illinois) was inoculated in Widdel and Bak freshwater medium (Widdel and Bak, 1992) with lactate (20 mM) and sulfate (28 mM). Growth was monitored through sulfide measurements (Cline, 1969) and microscopy. Strains were isolated from the enrichment using an agar dilution series in B3 medium with lactate (20 mM) and sulfate (28 mM). Single colonies were picked and transferred to fresh medium. Purity was ascertained through a second agar dilution series. Subsequent colonies were picked and transferred into Widdel and Bak freshwater medium with lactate (20 mM) and sulfate (28 mM). Contamination of cultures was screened by plating 100 μl of fully grown culture on TSA and R2A plates and incubating the plates both aerobically and anaerobically.

Physiological analysis

Growth rates at 20°C, 25°C, 30°C, 37°C, 40°C and 45°C were determined by measuring optical density at 600 nm (OD_{600}) on duplicate cultures grown in Widdel and Bak freshwater medium with lactate (20 mM) and sulfate (20 mM).

Phenotypic analysis of carbon source, electron acceptor and electron donor was performed using the Widdel and Bak base freshwater media composition amended with 20 mM of every electron donor and 10 mM of every electron acceptor. Cultures were incubated for 10 days at 37°C with a shaking speed of 200 r.p.m. Cultures were inspected microscopically for growth.

Phylogenetic analysis

DNA was extracted from the isolated strains using the FastDNA SPIN Kit for Soil (Qbiogene) as per the manufacturer's instructions. Polymerase chain reaction amplification of most of the 16S rRNA, the complete 16S-23S ITS and a small portion of the 23S rRNA gene was performed using the primers 8F (Lane, 1991) and BactLSU66R (Suzuki *et al*., 2004). Each 50 μl of PCR reaction contained 5.0 μl of 10× buffer, 0.5 μM of each primer, 0.25 μM dNTPs (10 mM of each nucleotide), 1.0 U of *Pfu*Ultra (Stratagene) and 1.0 μl of template. An initial denaturation step of 94°C for 2 min was followed by 30 cycles of 94°C per 30 s, 55°C per 30 s and 72°C per 3 min. This was followed by a final elongation step of 72°C for 5 min.

PCR products amplified were cloned using the Zero Blunt TOPO TA Cloning for Sequencing Kit (Invitrogen) and sequences were obtained using an ABI3730XL automated DNA sequencer. Individual contigs were then constructed using Sequencher (Gene Codes, Ann Arbor, MI). Phylogenetic relationships were screened using BLAST (Altschul *et al*., 1997) and CLUSTAL_X (Thompson *et al*., 1997).

Partial dissimilatory sulfite reductase (*dsrAB*) gene sequences were amplified by PCR using the primers DSR1F and DSR4R (Wagner *et al*., 1998). Reaction conditions were identical to those used for amplifying the 16S rRNA genes, except that an annealing temperature of 55°C was used**.** All amplified DSR-encoding genes were cloned and sequenced as described above.

Pulse-field gel electrophoresis

Pulse-field gel electrophoresis analysis of genomic DNA was conducted on a CHEF-DF III system (Bio-Rad Laboratories, CA). Genomic DNAs from strains Hildenborough and DePue were embedded in 1% SeaKem agarose, prepared and digested with either I-CeuI or NotI as per instrument instructions. Running conditions included the following: 0.5× TBE running buffer, 1% SeaKem agarose in 0.5× TBE, 10°C running temperature, a 120° switching angle, 4.0 V cm^{-1} for 36 h with a switching time linearly ramped from 30 to 120 s, followed by 14 h at 1.8 V cm^{-1} with a switching time linearly ramped from 120 to 1200 s.

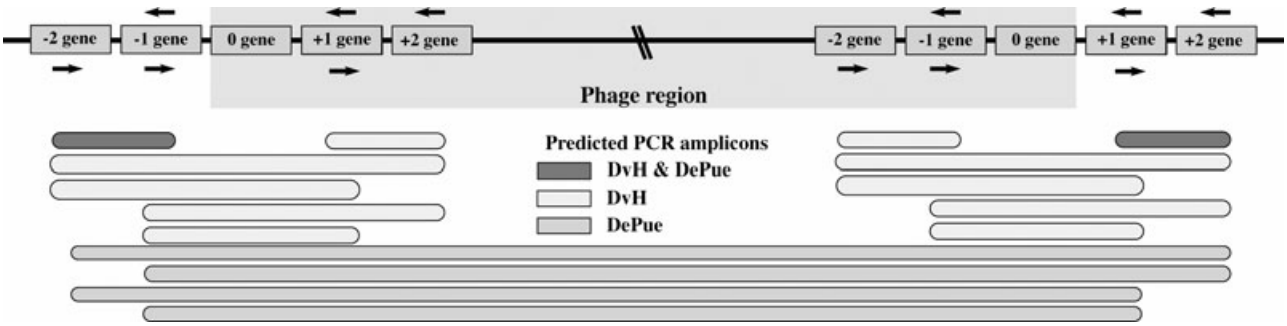

Fig. 5. Polymerase chain reaction amplification schematic for putative bacteriophage regions in strain DePue.

Microarray comparison between isolated strains and D. vulgaris *Hildenborough*

Concentrated cell pellets were lysed using TRIzol (Invitrogen) and chromosomal DNA extracted using phase separation. Purified DNA (500 ng) was labelled with Cy3 or Cy5, concentrated and hybridized as described previously (He *et al.*, 2005). Hybridized microarray slides were scanned and analysed as described previously using ScanArray Express microarray analysis system (He *et al.*, 2005). Scanned images were quantified using the software ImaGene 5.5 (Biodiscovery, CA). For each spot, the signal-to-noise ratio (SNR) was computed to discriminate true signals from noise. The SNR was calculated as follows: SNR = (Signal mean – Background mean)/(Background standard deviation), and an SNR of 3.0 was set as the threshold. For each ORF in the Hildenborough genome, if the ratio of signal intensity of Hildenborough to DePue was >0.5, the ORF was considered present in both genomes, and if the ratio was <0.5, the ORF was considered absent in the DePue genome, although almost all ratios were close to 1.0 or 0 in this experiment.

Polymerase chain reaction analysis spanning the putative phage-encoding regions

Primers were designed with homology to the *D. vulgaris* Hildenborough sequences for the two ORFs immediately flanking the six putative phage-encoding regions. Primers were designed either manually with ARB (Ludwig *et al.*, 2004) or with a commercial software packer (Rozen and Skaletsky, 2000). Additional primers were designed to phage-specific genes at the ends of the putative phage genomes (see Fig. 5 for primer schematic and Table 4 for primer sequences). Primer specificity was confirmed against *D. vulgaris* Hildenborough before attempts were made at amplifying across the putatively deleted genomic regions in strain DePue. Polymerase chain reaction conditions were identical to those described above, except that an annealing temperature of 59°C and an elongation time of 4 min were used. All amplified products were cloned and sequenced as described above.

Those regions in strain DePue successfully bridged by PCR were cloned and sequenced as described above. BLASTN comparisons against the *D. vulgaris* Hildenborough genome and the general use database determined the similarity of the DePue and Hildenborough strains, and the identity of sequences found solely in the DePue strain.

Phage isolation

Temperate phages were isolated from *D. vulgaris* Hildenborough by growing cultures on a liquid Yen45 medium containing 8 mM $MgCl_2 \cdot 6H_2O$, 20 mM NH_4Cl, 0.6 mM $CaCl_2 \cdot 2H_2O$, 2 mM phosphate buffer (K_2HPO_4/NaH_2PO_4, pH 7.4), 30 μM $FeCl_2$, 30 mM Tris-HCl (pH 7.4), 20 mM $NaHCO_3$, 60 mM lactate, 30 mM sulfate and 1.1 μM Ti-citrate. Vitamins (1 ml l^{-1}) (Widdel and Bak, 1992) and 2 ml l^{-1} trace metals (Widdel and Bak, 1992) (no NTA or $FeCl_2$) are added as well. After cultures reached post-stationary phase (approxi-

Table 4. Primer used for amplifying across putative bacteriophage regions.

Primer	Sequence (5′ → 3′)
R2b–2f	TCG CCC GAA CTC ATC GTC GAG AA
R2b–1f	TGA CGG CAC TTT GCG CTT GAC TG
R2b+1r	CTG GCG CAG GGG TGC ATC ATA AA
R2b+2r	CCG CTG AAC ATC GGC GGT TTC TT
R2e–2f	CCT GAT TGC AGA CGC CTA TCT GC
R2e–1f	GTC GAG CAA GTC CTT GCT GAT GG
R2e+1r	CAT CCA GTC CGC TTG CAA GCT CT
R2e+2r	GAT AGG GTG TCC GGG TGT TCA TG
R3b–2f	TAC GCG ATG CAC CAT GGG ACT TG
R3b–1f	GAT GTG GAA CGT ATC GCC GAC GA
R3b+1r	CAT CGG CGT ATT CAC CCG CAT TG
R3b+2r	GAC TCA ACG GCA CAC CTC AAT CC
R3e–2f	CTT ACG GAG GCT GAG AAG CTC GA
R3e–1f	GGC ATG AGC AGT GGA ACA GAG AG
R3e+1r	ACG CGC TCG ACT CGA AAT CGA GT
R3e+2r	ACA ACG ACC GCA AAC TAC CGG CT
R4b–2f	TCC TTG CGG CTG CAC TCT TCG AT
R4b–1f	TTG GAT AGA GCG AGC GCC TCC TA
R4b+1r	GGC AGA GCT TGA AGC GAT GGT TG
R4b+2r	AAT CGT TGC ACT CGC GTC ACG CA
R4e–2f	ATG TCC AGC CTC GCT GCA GAA GA
R4e–1f	AAG GTC TAC CCC ATC GGC AAG TC
R4e+1r	TGA GAC GCC AAG GAG ATG TGC TG
R4e+2r	GGC TAT GAG GCA TGG TTC CAG TC

Primer names were constructed as follows: R# → region number; b or e → beginning or end of putative bacteriophage region.

mately 4–10 days), approximately 0.1 ml of filtrate (0.22 μm pore-sized filter) was applied to a lawn of strain DePue prepared from mid-exponentially growing cultures. Approximately 30–500 plaques per ml were obtained. Phage stocks were created by plating 10^3–10^4 phage on a lawn of strain DePue cells growing on Yen45 medium. After 3–4 days (OD_{600} = 0.3–0.4), bacteriophage are induced by adding 8 μg ml^{-1} Mitomycin-C. After 20 h of induction at room temperature, the medium was removed from the plate surface and filtered (0.22 μm). The phage stocks were concentrated by ultracentrifugation at 100 000 *g* for 1 h and visualized. Scanning electron micrographs were obtained from the Electron Microscopy Core Facility at the University of Missouri-Columbia (Missouri).

Nucleotide sequences accession number

The sequences described in this article have been deposited in GenBank under Accession No. DQ826728-DQ826732.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. Microarray results of putative bacteriophage-region ORFs.

This material is available as part of the online article from http://www.blackwell-synergy.com

