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Development of a Novel Genetic System To Create Markerless Deletion Mutants of *Bdellovibrio bacteriovorus*▽

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Bdellovibrio bacteriovorus **is a species of unique obligate predatory bacteria that utilize gram-negative bacteria as prey. Their life cycle alternates between a motile extracellular phase and a growth phase within the prey cell periplasm. The mechanism of prey cell invasion and the genetic networks and regulation during the life cycle have not been elucidated. The obligate predatory nature of the *B. bacteriovorus* life cycle suggests the use of this bacterium in potential applications involving pathogen control but adds complexity to the development of practical genetic systems that can be used to determine gene function. This work reports the development of a genetic technique for allelic exchange or gene inactivation by construction of in-frame markerless deletion mutants including the use of a counterselectable marker in *B. bacteriovorus*. A suicide plasmid carrying the *sacB* gene for counterselection was used to inactivate the *strB* gene in *B. bacteriovorus* HD100 by an in-frame deletion. Despite the inactivation of the *strB* gene, *B. bacteriovorus* was found to retain resistance to high concentrations of streptomycin. The stability of a plasmid for use in complementation experiments was also investigated, and it was determined that pMMB206 replicates autonomously in *B. bacteriovorus*. Development of this practical genetic system now facilitates the study of *B. bacteriovorus* at the molecular level and will aid in understanding the regulatory networks and gene function in this fascinating predatory bacterium.**

Bdellovibrio is a small, motile gram-negative bacterium with an obligate predatory life cycle that utilizes other gram-negative bacteria in the predation process. The *Bdellovibrio* life cycle consists of two phases: a free-swimming extracellular attack phase and an intraperiplasmic phase in which the bacteria replicate by filamentous fragmentation in the periplasm of the prey cell. The growth phase ends with lysis of the prey cell by the *Bdellovibrio* progeny, which enter the free-swimming phase ready to invade other prey cells. *Bdellovibrio* is different from other predatory bacteria in that it enters the prey cell but remains in the periplasm. After the *Bdellovibrio* bacterium penetrates the prey cell, the prey cell remains intact but becomes rounded in shape. This prey cell, containing the growing *Bdellovibrio* bacterium, has been termed a bdelloplast. Interestingly, some strains of *Bdellovibrio* can generate mutant cells that are able to grow axenically and are referred to as host independent (1, 5). *Bdellovibrio* preys on a variety of other gram-negative bacteria, some of which are pathogens and thus have potential applications in either environmental or medical settings (19, 25, 29). The development of practical molecular genetic techniques that can be used for research involving gene function may lead to the discovery of novel antibacterial enzymes in this fascinating bacterium.

Despite the growing interest in this unique bacterium for potential applications, the mechanism of prey cell invasion and the genetic networks and regulation during the life cycle, as well as the mechanism underlying the phenotype change to host independence, are not understood. This lack of understanding of almost all aspects of *Bdellovibrio* is due in large part to the scarcity of work at the molecular level that has been reported. The obligate predatory nature of *Bdellovibrio* adds some complexity to the development of a feasible system for genetic manipulation to determine gene function in these bacteria. However, given the possible applications involving use of either live *Bdellovibrio* bacteria or enzymes encoded by the *Bdellovibrio* genome, it is important to continue to expand the repertoire of useful genetic tools for studying this bacterium at the molecular level.

Genetic work with *Bdellovibrio* was pioneered in 1992 by Cotter and Thomashow, who transferred plasmids to *Bdellovibrio bacteriovorus* strain 109J by using conjugal matings and determined that IncQ plasmids are autonomously replicated in *B. bacteriovorus* but that some IncP plasmids, including pVK100, are not (5). They then proceeded to clone an open reading frame (ORF) from *B. bacteriovorus* 109J into pVK100, which they then integrated by homologous recombination into the chromosomal DNA of the host-independent form of the *B. bacteriovorus* 109J strain harboring a mutation in that ORF (6). Phenotype characterization was carried out using the merodiploid, and no attempt to select for excisants was reported. Despite the integration of the wild-type gene in these merodiploids, they never returned to the fully wild-type phenotype that would be expected if an allelic exchange method had been available for use instead. It was over 10 years later, in 2003, that further molecular genetic work with *Bdellovibrio* was reported. Building on the earlier results showing that the IncP plasmid pVK100 cannot autonomously replicate in *B. bacteriovorus* and thus can be used as a suicide vector, Lambert and

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coworkers used the plasmid pSET151, which also has the IncP origin of replication, as a suicide vector (14). The plasmid pSET151 was used to independently inactivate the *mcp2* and *mviN* genes by insertion of alleles disrupted with a kanamycin (Km) resistance cassette on the chromosome of *B. bacteriovorus* strain 109J. Knockout mutants were obtained by screening for loss of the wild-type gene without the use of counterselection. This same method was used later to inactivate flagellin genes in *B. bacteriovorus* 109J (13).

Recently, the complete genome sequence of *B. bacteriovorus* strain HD100 was reported (19). With this genome sequence now available, a combination of bioinformatics and transcriptional analysis during different times in the *Bdellovibrio* life cycle can suggest specific ORFs that might be associated with a particular function of interest. Reverse genetic approaches can be used to determine gene function, and furthermore, in studying genes associated with the invasion process, development of genetic methods that can be used with *Bdellovibrio* to determine gene essentiality will become important. Thus far, chromosomal knockouts have been limited to insertion of a Km resistance cassette, and these mutations are not reported to be in-frame; therefore, downstream polar effects might have some effect on phenotype analysis. Furthermore, validation of phenotype change due solely to mutations in specific genes by complementation is an important issue to be addressed for *Bdellovibrio*. As mentioned, Cotter and Thomashow demonstrated that several IncQ plasmids replicate autonomously in *B. bacteriovorus* (5). This result suggests that these plasmids can be used for complementation experiments. In fact, more recent work by others made use of this information, whereupon pMMB206 (15), a plasmid with an IncQ origin of replication, was found to replicate autonomously in *B. bacteriovorus* and was used in RNA interference experiments to reduce expression of the MotA protein in *B. bacteriovorus* 109J (10). Despite another recent report that there is no suitable vector for complementation in *B. bacteriovorus* (13), we have been successful in maintaining pMMB206 extrachromosomally in both the HD100 and the B610 strains of *B. bacteriovorus* for over 4 months. The apparent discrepancy among reports pertaining to the stability of pMMB206 in *B. bacteriovorus* may be due to the low copy number of the plasmid.

Although gene inactivation by antibiotic cassette insertion was an important step forward, if a deeper understanding of the *Bdellovibrio* life cycle at the molecular level is to be gained, a genetic system in which markerless in-frame chromosomal deletion mutants can be constructed and complemented must be developed. In addition, for some experiments it may be desirable to replace the wild-type allele with a mutant allele rather than completely knock out a gene; thus, a means of allelic exchange rather than insertion inactivation is important. The evaluation of possible counterselectable markers that can be successfully used in *Bdellovibrio* bacteria has not been reported. One method of counterselection widely used with gram-negative bacteria is detection of sucrose sensitivity in the presence of the *Bacillus subtilis sacB* gene coding for levansucrase (20). This method of counterselection has been found to be useful for several other environmental bacteria, including *Geobacter* (4), *Desulfovibrio* (11), and *Myxococcus* (28) bacteria. However, *Leptospira*, another environmental bacterium, was found to be sensitive to sucrose; thus, counterselection using the *sacB* gene was not possible (18).

In the present work, we describe a practical technique for allelic exchange or gene inactivation by in-frame deletion and the use of a counterselectable marker in *B. bacteriovorus*. In choosing a gene to inactivate, only genes known to be nonessential for cell viability were considered. The *strB* gene, which is known to confer streptomycin (Sm) resistance in other bacteria (27), seems a logical choice since it is not essential for survival unless bacterial cultures are grown in Sm. Use of the *sacB* gene was found to be a valuable method for selecting double recombinants in *B. bacteriovorus*. A suicide plasmid, pSSK10, which is derived from pDS132 (17) by addition of a Km resistance cassette and which carries the *sacB* gene for counterselection and the RK6 origin of replication, was constructed. Using our constructed suicide plasmid, we have inactivated the *strB* gene in *B. bacteriovorus* strain HD100 by an in-frame deletion, showing for the first time in *Bdellovibrio* the ability to make markerless deletion mutants and to use a counterselectable marker. In addition, the stability of a plasmid for use in complementation experiments was investigated, and it was determined that pMMB206 replicates autonomously in *B. bacteriovorus*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Experiments reported in this work used *B. bacteriovorus* strain HD100 (gi 42494925) and strain B610 (7). The *Escherichia coli* strains and plasmids used are listed in Table 1. *E. coli* DH5α*λpir* or S17-1*λpir* was used for cloning manipulations involving the suicide plasmids, and either *E. coli* SM10*λpir* or *E. coli* S17-1*λpir* was used for conjugal matings since the plasmids used are based on the R6K origin of replication, thus requiring the product of the *λpir* gene for replication.

Media, growth conditions, and conjugation. *B. bacteriovorus* HD100 was grown in liquid cocultures along with *E. coli* prey by using dilute nutrient broth (DNB) consisting of 0.8 g/liter nutrient broth (Difco) supplemented with 2 mM $CaCl_2$ and 3 mM $MgCl_2$. Wild-type HD100 was typically maintained using *E. coli* ML35, and Km-resistant HD100 was grown in broth supplemented with Km (25 $\mu g\ ml^{-1}$), with *E. coli* SM10*λpir* as prey. To obtain isolated plaques, HD100 was plated using the double overlay method (14). Liquid cocultures were shaken and grown at room temperature, and plates were incubated at 30°C. Km (50 $\mu g\ ml^{-1}$) was added to both the top and the bottom agar for selection of merodiploid HD100. DNB medium prepared without the additional salts and supplemented with 5% sucrose was used for selection of chromosomal deletion mutants. All *E. coli* strains were grown at 37°C in Luria-Bertani (LB) media supplemented with Km (50 $\mu g\ ml^{-1}$), Sm (50 $\mu g\ ml^{-1}$), or chloramphenicol (Cm; 30 $\mu g\ ml^{-1}$) when appropriate. The suicide plasmid and the plasmid pMMB206 were transferred by conjugation to HD100 by using *E. coli* S17-1*λpir* and SM10*λpir* donor cells, respectively, according to the conjugal mating procedure previously described for *B. bacteriovorus* (5). Briefly, 12 ml of donor cells grown to the top of log phase was pelleted and resuspended in 100 μl DNB. Cocultures grown in 50 ml DNB were filtered and pelleted as described above, and the *B. bacteriovorus* cells were resuspended in 100 μl DNB and then spread on a nitrocellulose filter placed on a peptone yeast agar plate. The donor cells were then spread on the *B. bacteriovorus* cells, and the plate was incubated at 30°C for 22 h. After the mating, the filter was vortexed in 2 ml DNB and appropriate dilutions were plated in DNB supplemented with Km (50 $\mu g\ ml^{-1}$) by using the double overlay method described above.

Cloning, PCR, and RT-PCR. *Pfu* Turbo DNA polymerase (Stratagene) was used for all cloning and sequencing PCRs. A QIAprep spin mini prep kit (QIAGEN) or a plasmid midi kit (QIAGEN) was used for plasmid extractions. Bacterial pellets for both HD100 RNA and genomic DNA extractions were prepared free of contaminating *E. coli* prey cells by first filtering the cocultures through a 0.8-μm syringe filter and then two times through 0.45-μm syringe filters, which allow the smaller *B. bacteriovorus* cells but not the *E. coli* prey cells to pass. The absence of prey cells in the filtrate, as well as the absence of donor cells after experiments involving conjugation, was confirmed by incubation of an aliquot of the filtrate on LB media overnight. DNA was extracted from *B.*

TABLE 1. *Escherichia coli* strains and plasmids used in this study

Strain or plasmid	Relevant features	Reference or source
E. coli strains		
DH5α	F$^-$ φ80*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_k^- m_k^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Gibco-BRL
ML35	*lacI lacY*	21
DH5αλ*pir*	DH5α with λ*pir* lysogen	M. Donnenberg
SM10λ*pir*	*thiL thrL leuB6 supE44 tonA21 lacY1 recA*::RP4-2-Tc::*Mu* Km^r	23
S17-1λ*pir*	*pro recA thi hsdR* Hfr RP4-2 (Tc::*Mu*) (Km::Tn*7*) Sm^r Tp^r λ*pir* lysogen	24
Plasmids		
pDS132	*R6K ori mobRP4 cat sacB*	17
pCR2.1-TOPO	Source of Km^r	Invitrogen
pSSK10	Derived from pDS132; insertion of Km^r along with NdeI, MluI, and XhoI cloning sites	This work
pSSK20	Δ*strB* cloned into pSSK10	This work
pMMB206	IncQ *lacI*^{q} P*taclac lacZ*α *rrnB cat mobRP4*	15
pMMB0307	pMMB206 with *strB* cloned	This work
pACYC184	Cm^r	New England Biolabs

bacteriovorus pellets by using a DNeasy tissue kit (QIAGEN). All sequences were obtained with primers indicated in Table 2. Specifically, the *strB* gene was sequenced from genomic HD100 DNA by using primers Bd0307F and Bd0307R, and the *rpsL* gene was sequenced using primers Bd2981F and Bd2981R. All sequencing reactions were carried out using a BigDye Terminator cycle sequencing kit (Applied Biosystems) and an ABI 3730xl automatic sequencer. For PCR screening, reaction mixtures of *B. bacteriovorus* in cocultures with puRe *Taq* ready-to-go PCR beads (Amersham Biosciences) were used. For these reaction mixtures, 1.6 ml of clear coculture was pelleted in a microcentrifuge tube at maximum speed for 30 min at 4°C. The pellet was resuspended in 100 μl H_2O, and the cell suspension was boiled for 5 min, after which 10 μl was used in a 25-μl-total-volume PCR mixture. With all sets of primers used, the absence of a PCR product for only *E. coli* prey cells confirms amplification of only *B. bacteriovorus* DNA in the pellets prepared from the cocultures. RNA was stabilized in the filtered *B. bacteriovorus* cultures before pellet preparation using RNAprotect bacterial reagent (QIAGEN), and RNA was extracted from the pellets using an RNeasy kit (QIAGEN). Possible contaminating DNA was removed by RQ1 RNase-free DNase (Promega) treatment followed by a cleanup with the RNeasy kit. Absence of DNA was determined by lack of PCR product by using universal primers for the 16S rRNA gene and HD100 DNA as a positive control. Two primers for the *strB* gene, RTStrBF (outside the deleted region in the Δ*strB* mutant) and RTStrBR (within the deleted region in the Δ*strB* mutant), were designed, along with two primers for the *rpsL* gene, rpsLF2 and rpsLR, as a load control. These primer sets were tested using both HD100 and *E. coli* ML35 DNA to show specificity for HD100 by the absence of a PCR product for the *E. coli* DNA. Reverse transcription (RT)-PCR was performed with 200 ng of total RNA extracted from wild-type HD100 and the HD100Δ*strB* mutant by using a OneStep RT-PCR kit (QIAGEN), along with *E. coli* ML35 RNA as a control for further confirmation of the absence of prey cell RNA contributing to the RT-PCR product from the extracted HD100 RNA.

TABLE 2. Primers used in this work

Primer	Sequence (5′ to 3′)[a]
Bd0307F	GCCAATCTGGATCAAGGTC
Bd0307R	GCTGTTTTCTGTCAGTGGTC
Bd2981F	GAAATGTAAGTTCCAGCGTC
Bd2981R	AACTTAGCAATTACTAGATCCTTG
PTPKRF	ATAAGAGCTCCTCGAGACGCGTCATATGG GACAAGGGAAAACGCAAG
PTPKRR	ACTAGAGCTCCGACACGGAAATGTTGAAT ACTC
PDS132F	CTGTTGCATGGGCATAAAG
PDS132R	AGGAACACTTAACGGCTGAC
STRBF1	ATAGTAACATATGGCAAAGTGGCGGAGC TGAC
STRBR1	AATAGCATGCCGTCAGATCCCAGCGCAG
STRBF2	TGATGCATGCTCTGCAGCCTGGAGCGTG
STRBR2	ATACTCGAGAGTAACGGTACTGGGGCGTC
SBF	TATTAACCTTTACTACCGCACTG
SBR	GTCCTTGTTCAAGGATGCTG
P206F	ATGATAGCCTACGAGACAGCAC
P206R	GCTGCGCTAGGCTACACAC
P206R2	TTGAGAATATGTTTTTCGTCTCAG
SBcomR	TATCCCGGGATCCATTGAGCGTTGGTG
SBcomF	TATAAGCTTCCAGAGGATTTTCATTCAAC
PB206F	CGTATGTTGTGTGGAATTGTGAG
PB206R	GATTAAGTTGGGTAACGCCAG
RTStrBF	ATTTTCCCGCACCGTTC
RTStrBR	GCAAGAAGTCACGCACATG
rpsLF2	CATCAAAAGTGAGCGTACTG
rpsLR	CGCAGACGACCGTTTACAC

[a] Restriction enzyme sites are underlined.

Construction of the suicide plasmid pSSK10. A suicide vector for making deletion mutants of *B. bacteriovorus* was constructed by insertion of a Km resistance cassette along with additional unique restriction enzyme sites into pDS132, a suicide vector with the R6K origin of replication that carries the *cat* gene for selection of integrants and the *sacB* gene for counterselection of excisants (17). The Km resistance cassette including the promoter was amplified from pCR2.1-TOPO by using primers PTPKRF and PTPKRR, which include SacI sites. Primer PTPKRF also includes unique sites NdeI, MluI, and XhoI, which can be used for cloning. The PCR-amplified fragment containing the Km resistance cassette was blunt end ligated into the unique SacI site in pDS132, resulting in the plasmid pSSK10. The orientation of the Km resistance cassette was determined by digestion and confirmed by sequencing using primers PDS132F and PDS132R.

Construction of HD100 in-frame *strB* deletion mutant. The *strB* gene (locus tag Bd0307), which is known to confer Sm resistance in other bacteria (27), was knocked out by allelic exchange using an in-frame deletion cloned on the suicide plasmid pSSK10. Construction of the *strB* knockout suicide vector pSSK20 was accomplished by triple ligation of the two flanking regions with the pSSK10 plasmid digested with NdeI and XhoI. One set of primers, STRBF1 and STRBR1, was designed to include the first 40 amino acids of the StrB protein, and a second set of primers, STRBF2 and STRBR2, was used to produce a PCR product including the 28 C-terminal amino acids. These two PCR products were each ~700 bp and had an SphI site included on the primers within the *strB* gene that was used for the triple ligation. The correct construction was confirmed by sequencing. *E. coli* S17-1λ*pir* harboring pSSK20 was used as a donor for conjugation into the recipient HD100. The exconjugates were plated with *E. coli* SM10λ*pir* prey on plates containing no antibiotic, in addition to the plates containing Km (50 μg ml^{-1}), to determine the frequency of integration of the suicide plasmid. Eight of the plaques grown under Km selection were picked and transferred to liquid cocultures in DNB supplemented with Km (25 μg ml^{-1}). Upon clearance, the presence of merodiploid HD100 in the cocultures was confirmed by PCR using the same outer flanking primers that had been used for cloning. Further corroboration of the integration of the suicide plasmid was

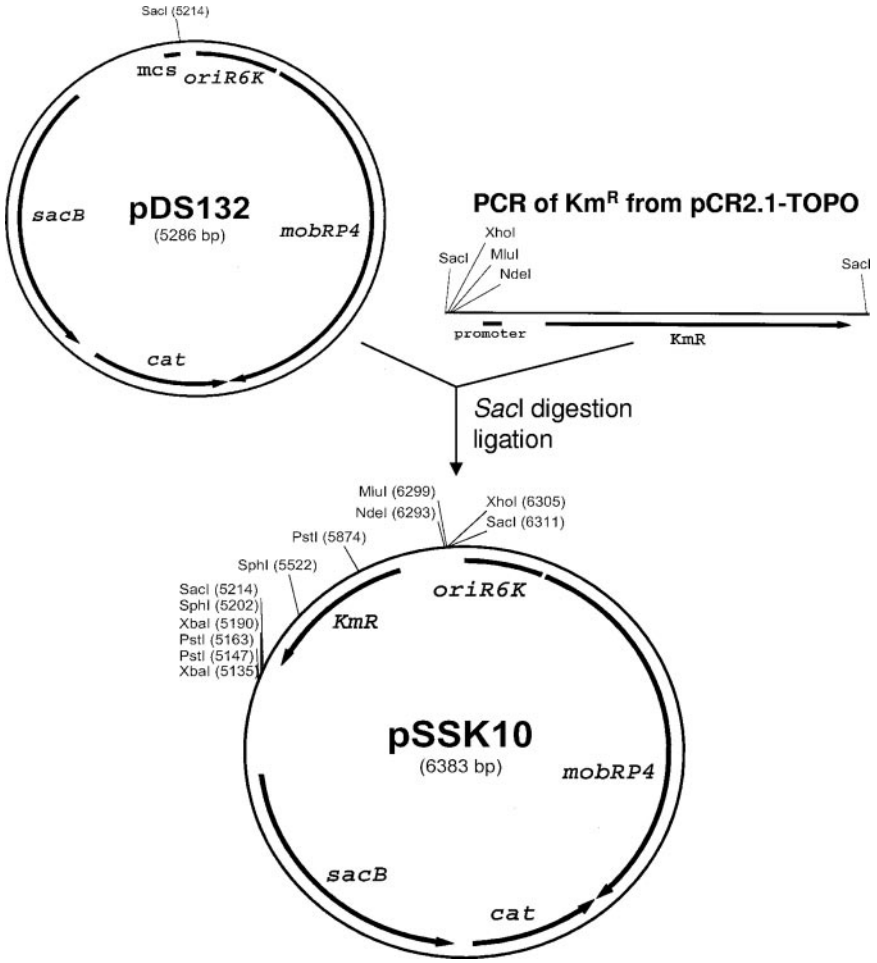

FIG. 1. Construction of the suicide plasmid pSSK10. The plasmid pSSK10 was derived from pDS132 by addition of the Km^r cassette from pCR2.1-TOPO and unique restriction enzyme sites NdeI, MluI, and XhoI. The PCR product containing the Km^r cassette was obtained with primers having terminal SacI sites and was ligated into pDS132 at the unique SacI site.

obtained by PCR amplification of the *sacB* gene using primers SBF and SBR. An aliquot of a merodiploid culture was pelleted, the pellet was washed with DNB, and then the cells were added to fresh DNB with no antibiotic and *E. coli* ML35 prey. This coculture was grown for 48 h to allow for excision of the suicide plasmid by a second homologous recombination event. An aliquot of this coculture was transferred to DNB broth containing 5% sucrose and fresh prey cells to select for excisants. After clearing of the cocultures, dilutions were plated and 40 plaques were picked into DNB broth with fresh prey. These cocultures were then screened by PCR for knockout mutants using the outer flanking primers STRBF1 and STRBR2. The DNA was extracted from one of the *B. bacteriovorus strB* knockout mutants for confirmation of the correct in-frame deletion of the *strB* gene on the chromosome by sequencing.

Confirmation of the stability of pMMB206 in *B. bacteriovorus*. Two *B. bacteriovorus* strains, HD100 and B610, were used for experiments to confirm the autonomous replication of pMMB206 in *B. bacteriovorus*. The plasmid pMMB206 was transferred to HD100 and B610 by conjugation using *E. coli* SM10λ*pir* donor cells as described earlier (5). *E. coli* SM10λ*pir* made Cm resistant by transformation with pACYC184 was used as prey for plating the exconjugates. A single plaque of exconjugate HD100 or B610 was picked and grown in liquid media supplemented with Cm (10 μg ml^{-1}). DNA was extracted from the Cm-resistant *B. bacteriovorus* after filtration to remove the prey and analyzed by PCR for the presence of the plasmid pMMB206. Two sets of primers that give PCR product sizes of 514 bp and 1,323 bp with pMMB206 as a template but do not result in PCR products from the plasmid pACYC184 were used. These primer sets consisted of one forward primer, P206F, paired with two different reverse primers, P206R and P206R2. Wild-type HD100 or B610 DNA was used as a negative control. In order to definitively confirm that the *B. bacteriovorus* bacteria were carrying pMMB206 extrachromosomally, the extracted B610 DNA was used for transformation into *E. coli* DH5α. The HD100 strain harboring pMMB206 was transferred weekly and filtered numerous times for 4 months in DNB containing Cm (10 μg ml^{-1}). To rule out the possibility that any donor *E. coli* SM10 λpir cells were still present in the cocultures even after the filtering process, the HD100 strain carrying pMMB206 was transferred twice in DNB media containing ampicillin (Amp; 100 μg ml^{-1}) in addition to the Cm. DNA was then extracted from the *B. bacteriovorus* bacteria and used for transformation into *E. coli* DH5α. Following each of the transformations, a colony was grown and the plasmid was extracted. The size of the extracted plasmid was compared to that of pMMB206 by gel electrophoresis, and the extracted plasmid was employed as a template for PCR, using the primers described above that are specific for the pMMB206 plasmid.

Cloning of the *strB* gene on pMMB206. The *strB* gene, along with 236 bp upstream, was amplified from the wild-type HD100 genomic DNA by PCR using primers SBcomF and SBcomR, which included SmaI and HindIII restriction sites for directional cloning into the plasmid pMMB206, to create pMMB0307. This construction was transformed into *E. coli* DH5α, and after confirmation of the correct sequence using primers PB206F and PB206R, located on either side of the cloning site, a clone was tested for Sm resistance. The plasmid pMMB0307 carries the *cat* gene and the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible P*taclac* promoter upstream of the cloned fragment. Therefore, the HD100 StrB protein function in the *E. coli* DH5αpMMB0307 clone was tested on LB plates supplemented with Cm, 0.1 mM IPTG (Amresco), and Sm at either 0, 5, 10, or 50 μg ml^{-1}.

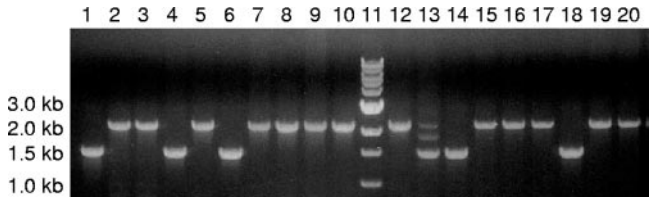

FIG. 2. PCR screening of exconjugate HD100. PCR products obtained using primers STRBF1 and STRBR2 (outer primers used for cloning the flanking regions of the *strB* gene deletion into the suicide vector) were visualized in agarose gels. This gel, one of several, shows 19 of the 40 plaques screened and includes 13 wild-type revertants, 1 merodiploid (lane 13), and 5 knockout mutants (lanes 1, 4, 6, 14, and 18). Lane 11 contains a molecular size marker.

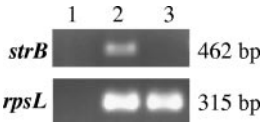

FIG. 3. RT-PCR analysis of wild-type HD100 and the *strB* mutant. Total RNA was extracted from *E. coli* ML35 (lane 1), wild-type HD100 (lane 2), and HD100Δ*strB* (lane 3) and used in RT-PCRs with primers specific for the *strB* gene. The *rpsL* gene was used as a load control for *B. bacteriovorus* RNA. The absence of an RT-PCR product in lane 1 indicates that the product seen for both the *strB* and the *rpsL* genes corresponds to *B. bacteriovorus* RNA rather than any possible contaminating *E. coli* RNA.

RESULTS

Construction of the knockout suicide plasmid pSSK20. Several clones resulting from the use of blunt-end ligation of the Km resistance cassette into pDS132 to derive pSSK10 were screened for the orientation of the inserted fragment. All clones had the same orientation, which is shown in Fig. 1; thus, this plasmid construction was used to clone for the *strB* knockout. The shortage of restriction enzyme sites available for cloning on pDS132 was at least partly alleviated by insertion of the additional NdeI, MluI, and XhoI sites cloned with the Km resistance cassette into pSSK10. Two of these sites were subsequently used for cloning the flanking regions for the in-frame deletion. The construction results in a deletion of 231 amino acids from the 299-amino-acid StrB protein.

Construction of HD100 in-frame *strB* deletion mutant. A comparison of the numbers of plaques obtained from exconjugates plated with and without Km selection showed that integration of the suicide plasmid took place at a frequency of about 10^{-6}. Since it was desired to use the *sacB* gene for counterselection of excisants, wild-type HD100 was tested for sucrose sensitivity and then for the best concentration of sucrose to use for the counterselection. Concentrations of 2%, 5%, and 10% sucrose (wt/vol) were screened. Although *B. bacteriovorus* bacteria were found to be viable in 10% sucrose, the coculture takes significantly longer to clear. Counterselection was, therefore, attempted using 5% sucrose plates but resulted in plaques corresponding to predominantly merodiploids. As a result, selection of excisants was accomplished in this work using liquid 5% sucrose media rather than sucrose plates. With this method, the cocultures were observed to clear in 4 days (typically, cocultures of HD100 clear in 2 days when transferred using DNB media with no sucrose). Of the 40 plaques screened via PCR after sucrose selection, only one merodiploid was found, thus showing that sucrose counterselection is an efficient method for use with *B. bacteriovorus*. Further substantiating this method of counterselection for *B. bacteriovorus*, during the selection process in constructing a deletion mutant of a different gene, over 120 plaques were screened for the second homologous crossover event and all were found to be excisants (S. R. Steyert and S. A. Pineiro, unpublished results). In the present work, 26 of the 40 plaques screened by PCR were found to contain wild-type revertant *B. bacteriovorus* and 13 corresponded to *strB* knockout mutants. An agarose gel representative of the results of the PCR screening for 19 of the plaques is shown in Fig. 2.

Confirmation of the *strB* gene in-frame deletion. DNA from one of the HD100Δ*strB* mutants was extracted and sequenced to confirm that the deletion was in-frame as constructed. As further confirmation of the deletion, RT-PCR products can be seen for the *strB* gene during the free-swimming extracellular stage in the life cycle in wild-type HD100 but not in the HD100Δ*strB* mutant. Figure 3 shows the presence of a transcription product in the wild-type HD100 isolate (Fig. 3, lane 2), in contrast to the mutant for which there is no product (Fig. 3, lane 3). In addition, the HD100Δ*strB* mutant was tested for Sm sensitivity but was found to retain resistance to high concentrations of Sm (>2,000 μg ml^{-1}). Since the *strB* gene was shown by RT-PCR to be transcribed in wild-type HD100, however, we questioned the functionality of the StrB protein. The DNA sequence of the *strB* gene in the HD100 isolate from our culture collection (EF493834) was compared to the *strB* gene sequence of the published HD100 genome (19) and was found to have 12 nucleotide differences. Some of these base pair changes are silent mutations, but the protein sequence does have four amino acid changes. Presumably, these mutations have occurred during propagation of HD100 in the laboratory under nonselective conditions. It is worth noting that the 16S rRNA gene sequence, as well as sequences of other loci from this strain, shows 100% identity with the GenBank sequences (data not shown). The functionality of the StrB protein in our HD100 strain was tested with *E. coli* DH5α carrying the plasmid pMMB0307, which contains the HD100 *strB* gene and promoter region. It was found to be sensitive to Sm concentrations of 5 μg ml^{-1}; therefore, the *strB* gene did not appear to confer Sm resistance in *E. coli* and thus presumably not in *B. bacteriovorus* either. The high level of Sm resistance that the *strB* deletion mutant was found to retain suggested the possibility of a mutation in the *rpsL* gene in our HD100 isolate. Mutations in the *rpsL* gene encoding the ribosomal protein S12 have been shown to confer Sm resistance in many bacteria (3). Therefore, the *rpsL* gene in our HD100 isolate was sequenced (EF493835) and this sequence compared to the published *rpsL* gene sequence (19). A BLASTp (http://www.ncbi.nlm.nih.gov/blast/) search of the DNA sequences reveals point mutation K42R (*E. coli* and *Bdellovibrio* numbering followed), which is an *rpsL* mutation commonly found to result in high levels of Sm resistance (3).

Stability of pMMB206 in *B. bacteriovorus*. The stability of the plasmid pMMB206 for use in complementation experiments was tested with the two *B. bacteriovorus* strains HD100

and B610. The plasmid pMMB206 was found by PCR to be present in the extracted DNA from both the HD100 and the B610 *B. bacteriovorus* strains, showing that this plasmid was successfully transferred into both strains. The extracted plasmids recovered from transformation of the DNA into *E. coli* DH5α were found in both cases to be the same size as pMMB206, and the two expected PCR products were obtained (data not shown). This confirms that pMMB206 is stably maintained extrachromosomally by autonomous replication in *B. bacteriovorus* and can be used for complementation experiments.

DISCUSSION

From the time of the sequencing of the *B. bacteriovorus* HD100 genome (19), there has been a renewed interest in the investigation of this predatory bacterium at the molecular level. Various aspects of the growth and development of *B. bacteriovorus* during its unique life cycle have been investigated biochemically and microscopically in the past, with a few more-recent molecular investigations, as is outlined in a recent review (12). The annotation of the *B. bacteriovorus* genome reveals the existence of many hydrolytic enzymes (19), some of which have no homology with known enzymes and may be involved in the unique predatory nature of the *Bdellovibrio* life cycle. In order to make further advances in determining gene function or elucidating the regulatory networks in *Bdellovibrio*, tools for more-efficient genetic manipulation are necessary.

The first suicide plasmid used in *B. bacteriovorus* was a cosmid, pVK100, belonging to the IncP incompatibility group, which does not replicate in *B. bacteriovorus* (6) and which carries antibiotic resistance cassettes for Km and tetracycline (Tc) as markers for selection. The large size of this plasmid makes it difficult to work with. More-recent genetic work with *B. bacteriovorus* has made use of another IncP plasmid, pSET151, as a suicide plasmid (13, 14). This plasmid has a more manageable size of 6.2 kb and a reasonable selection of cloning sites but lacks a useful counterselectable marker (2). An additional drawback to the use of this plasmid is that the marker for selection of plasmid integration after the first homologous recombination is Amp resistance. We have found *B. bacteriovorus* HD100 to be resistant to Amp at concentrations up to at least 100 μg ml^{-1}. In agreement, Cotter and Thomashow were unable to use either Tc or Amp to select for conjugal transfer of plasmids but were able to use Cm or Km (5). Use of pSET151 as a suicide plasmid was still possible because gene inactivation was carried out by insertion of a Km resistance cassette which could be used for selection of integrants. The problem of no counterselection was circumvented by resorting to the less efficient method of relying solely on screening by Southern blotting.

An alternative to using a plasmid with the IncP origin of replication as a suicide plasmid in *Bdellovibrio* is to use a plasmid with the RK6 origin of replication. Plasmids with the RK6 origin of replication are dependent on the π protein supplied in *trans* by the product of the λ*pir* gene for replication (8, 17). The plasmid pCVD442, which has been used successfully as a suicide vector in other gram-negative bacteria, is based on the RK6 origin of replication and carries the *sacB* gene for counterselection (8). However, pCVD442 uses Amp resistance as a marker for integration and thus cannot be used without modification for work with *B. bacteriovorus*. A derivative of pCVD442 carrying the *cat* gene for Cm resistance selection in place of Amp resistance as well as having the insertion sequences found in pCVD442 removed has been reported (17). This plasmid, pDS132, was used to construct the suicide vector used in this work. Our initial work using Cm to select for *B. bacteriovorus* containing the chromosomally integrated plasmid was unsatisfactory due to the fact that low Cm concentrations were necessary to maintain viability and reasonable times for coculture clearing, and this would lead to the appearance of some plaques on plates of exconjugates due to the wild-type HD100 strain. The other antibiotic to which *B. bacteriovorus* bacteria are sensitive and that has been used successfully is Km; thus, we decided to use Km selection by addition of a Km resistance cassette to pDS132. We also included three unique restriction enzyme sites that can be used to clone knockout constructions directly into the suicide vector.

Counterselection based on the *sacB* gene was found to be an efficient method for *B. bacteriovorus*, provided that the selection is done in liquid culture. Because of the added complexity of growing the prey bacteria with the *Bdellovibrio* bacteria, ideal concentrations of antibiotics to use for selection can be different depending on whether plates or liquid culture are used. Interestingly, attempts to pick merodiploid plaques into DNB broth with fresh prey supplemented with Km at the concentration of 50 μg ml^{-1} resulted in either no viable *B. bacteriovorus* bacteria or the need for an unreasonably long period of time for the coculture to clear even though plaques were observed to start growing in the plates within 2 days. Therefore, plaques were picked into DNB containing Km at a concentration of 25 μg ml^{-1}, which was determined to be sufficient to maintain the *B. bacteriovorus* bacteria as merodiploids. It appears that the lawn of prey growing in the soft agar has a protective effect on the *Bdellovibrio* bacteria possibly because it can immediately prey on the *E. coli* bacteria rather than swim to find the prey as it does in liquid coculture.

The *strB* gene codes for an aminoglycoside phosphotransferase, APH(6), usually linked upstream with another aminoglycoside kinase gene, *strA*, which confers Sm resistance. The linked *strA-strB* genes are found within the transposon Tn*5393* on large conjugative plasmids in bacterial plant isolates or on small nonconjugative broad-host-range plasmids in human clinical isolates (27). *B. bacteriovorus* is unusual in that it possesses no *strA* gene, but rather, a gene coding for carboxypeptidase and oriented antisense to *strB* is upstream of *strB*. Just downstream of the *strB* gene is an ORF annotated as a hypothetical protein. No ORF with homology to the *strA* gene is reported to be in the genome sequence of HD100 (19). Since we were successful in creating an in-frame deletion in the *strB* gene, we proceeded to test our deletion mutant to determine the level of Sm resistance that the gene confers in *B. bacteriovorus*.

The HD100Δ*strB* mutant retains a high level of Sm resistance. Sequencing of the *rpsL* gene in our wild-type HD100 strain showed that this high level of Sm resistance is most likely due to a common point mutation, K42R, known to confer Sm resistance at >2,000 μg ml^{-1} in other bacteria (3). This mechanism of Sm resistance is consistent with the observation that resistance was retained in the Δ*strB* mutant. Also, it cannot be

ruled out that the lack of permeability of the *B. bacteriovorus* outer membrane might account for some of the Sm resistance. We reasoned that if the StrB protein contributes significantly to the level of resistance to Sm in the wild-type HD100 strain, then observing a phenotype change in the deletion mutant followed by complementation would still be possible. The *strB* gene, along with an upstream sequence, had been cloned into pMMB206 for complementation. In order to determine whether the StrB protein is functional and, if so, what level of Sm resistance it confers, we tested the sensitivity to Sm of *E. coli* DH5α harboring this plasmid. We have determined by RT-PCR that the *strB* gene is transcribed, but it appears that the StrB protein is either not expressed or not functional, since *E. coli* DH5α bacteria harboring this plasmid were not viable in Sm concentrations as low as 5 $\mu g\ ml^{-1}$. This result is not completely surprising, given that there is certainly no selective pressure to keep a functional allele under normal conditions in which HD100 is maintained in the laboratory, and especially in our strain with an *rpsL* mutation.

We have shown that the plasmid pMMB206 autonomously replicates in two different *B. bacteriovorus* strains. This is evidenced both by the ability to continuously grow and transfer these *B. bacteriovorus* bacteria harboring pMMB206 in media supplemented with Cm at a concentration of 10 $\mu g\ ml^{-1}$ (in which wild-type HD100 and B610 are not viable) and by the recovery of pMMB206 from the extracted DNA. This result is consistent with the report that not only was green fluorescent protein expression detected in *B. bacteriovorus* strain 109J harboring pMMB206 carrying the *gfp* gene, but a phenotype difference in life cycle duration as well as altered motility was observed in 109J harboring pMMB206 with antisense *motA* cloned (10). Therefore, complementation using the plasmid pMMB206 in *B. bacteriovorus* appears feasible. Since a phenotype change in the HD100Δ*strB* mutant could not be detected, the mutant was not complemented.

Bdellovibrio is an obligate predatory bacterium; thus, any study attempting to determine at the genetic level how the *Bdellovibrio* bacterium invades the prey cell and how its life cycle is regulated will presumably involve investigation of genes that are essential for the viability of the *Bdellovibrio* bacterium. Therefore, there is a possibility of attempts to construct deletion mutants of essential genes. Different variations of gene essentiality experiments with other organisms have been reported (9, 16, 22, 26). One involves introducing a wild-type copy of the gene to be deleted on an autonomously replicating plasmid carrying a counterselection marker different from that used on the suicide plasmid into the strain merodiploid for the knockout. The wild-type *rpsL* gene acts in a dominant-negative manner to confer Sm sensitivity to the cell and has been used as a counterselection marker (20). Therefore, the HD100 *rpsL* Δ*strB* mutant that we have constructed would be ideal for use in gene essentiality studies using a wild-type copy of the *rpsL* gene cloned into pMMB206 as a counterselection marker.

Now that we have developed a system for allelic exchange or construction of in-frame markerless deletion mutants of *B. bacteriovorus* HD100, this bacterium can be genetically manipulated to test gene function. Exciting new investigations can be undertaken to study many aspects of *Bdellovibrio*, from chemotaxis in the free-living stage of the life cycle to metabolic changes within the bdelloplast as well as functional characterization of hydrolytic enzymes. Furthermore, the genetic system described for constructing mutants in the host-dependent *Bdellovibrio* bacterium can be easily modified for purposes of genetic manipulation in the host-independent phenotype. Importantly, application of the methods described in this work coupled with transcriptional analysis will aid in understanding the regulatory networks in this unique prokaryotic predator bacteria.

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