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GENETIC ANALYSIS OF HOST-INDEPENDENT MUTANTS OF BDELLOVIBRIO BACTERIOVORUS 109J

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GENETIC ANALYSIS OF HOST-INDEPENDENT MUTANTS OF *BDELLOVIBRIO BACTERIOVORUS* 109J

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ABSTRACT

GENETIC ANALYSIS OF HOST-INDEPENDENT MUTANTS OF *BDELLOVIBRIO BACTERIOVORUS* 109J

By

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Members of the genus *Bdellovibrio* are obligate intraperiplasmic parasites of other gram-negative bacteria. Certain spontaneous *Bdellovibrio* mutations eliminate the host cell requirement, giving rise to host-independent (H-I) mutants that can grow axenically in media containing peptone and yeast extract. This dissertation describes the genetic analysis of three such mutants derived from *B. bacteriovorus* 109J. In order to allow for such an analysis, a system for the genetic manipulation of *Bdellovibrio* was developed. In Chapter 1 I describe the conjugal transfer of broad host range cloning vectors, namely IncP and IncQ derivatives, from *E. coli* to *B. bacteriovorus*. IncQ derivatives were maintained in *B. bacteriovorus* via autonomous replication. IncP derivatives could only be maintained after integration, through recombination between cloned bdellovibrio sequences and the recipient genome. This system was used to deliver an IncP based wild-type *B. bacteriovorus* 109J genomic library into an H-I mutant, BB5, resulting in the identification of wild-type sequences that significantly enhanced plaque formation by the H-I mutant. These "enhanced-plaques" were dramatically larger and clearer than those formed by BB5, and were very similar to those formed by the wild-type except for a moderate reduction in diameter. Further genetic analysis, described in Chapter 2, narrowed the plaque-enhancing sequences down to a 959 bp *Eco*RI-*Xba*I fragment, and showed that the equivalent region in BB5 contained a mutation. Two additional H-I mutants were isolated and shown to carry mutations in the same 959 bp fragment. Because of its affect on plaquing ability, and the occurrence of mutation in three independent H-I mutants, this locus was termed *hit*,

for host interaction. Merodiploid recombinants that contained both wild-type and mutant derived *hit* sequences displayed "intermediate" phenotypes. BB5 recombinants that contained wild-type *hit* sequences displayed the enhanced-plaque phenotype, but still formed colonies that were indistinguishable from BB5. The reciprocal experiment, where mutant *hit* sequences were recombined into the wild-type, gave similar but slightly different results. These recombinants formed plaques that were very similar to wild-type, and also displayed a capacity for "density-dependent" axenic growth. These results are considered with respect to the genetic basis for host-independent growth.

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INTRODUCTION

Bdellovibrio Biology

The genus *Bdellovibrio*, originally described by Stolp in 1962 (24), comprises a group of gram-negative bacteria that are obligate intraperiplasmic parasites of other gram-negative bacteria. Bdellovibrios are small (approximately 0.3x1.5 um) curved rods that are highly motile by means of a single polar flagellum. Members of this genus are widespread, having been isolated from soil, fresh water, marine water and sewage.

The normal *Bdellovibrio* life cycle is bi-phasic, consisting of a free living search phase and an intraperiplasmic growth phase. While in search phase, the free swimming bdellovibrio actively seeks a suitable host, but does not multiply. A host cell encounter is an apparently random event, where the rapidly swimming parasite collides with, and attaches to, the host outer surface. Initial attachment is reversible, but is followed by irreversible attachment if the attack is to be succesful (34). The basis for host specificity is unknown; a particular bdellovibrio strain may parasitize a broad or narrow range of gram-negative species (25, 26).

Approximately 10 min after attachment, a bdellovibrio rapidly breaches the host outer envelope and becomes lodged within the host periplasm (34). Concomitant with entry, the host cell usually rounds up into a sphere. The rounded cell containing the bdellovibrio has been termed the bdelloplast. The mechanism for penetrating the host outer membrane is unknown. However, penetration of the host peptidoglycan is thought to be caused by bdellovibrio directed enzymatic activities (27, 31). The

primary activity is that of a glycanase, which solubilizes glucosamine moieties present in the host peptidoglycan and LPS. The appearance of this activity correlates with the time of entry, and is thought to create a localized hole in the host peptidoglycan. While most bdellovibrios express the glycanase activity, an exception is *B. bacteriovorus* strain W, which gains entry without any detectable glycanase (31). In this case a peptidase activity that solubilizes diaminopimelic acid residues is thought to allow entry by reducing the cross-linking of host peptidoglycan. A more detailed review of these and other activities directed against the host peptidoglycan can be found elsewhere (28, 31).

Also during the period of attachment and entry, the attacking bdellovibrio causes the cessation of metabolic activity within the host cell. Eleven min after attachment, the host cell has lost the ability to synthesize protein, RNA and DNA (32). Within 30 min after attachment the host cell can no longer respire exogenous substrates and its cytoplasmic membrane becomes permeable to small molecules (16). Neither the order of these events nor the mechanisms by which they occur are known.

At some point during the early stages of attachment and penetration, the parasite undergoes a transition from search phase to growth phase. After this transition, a bdellovibrio spends the initial 60 min of a 3-4 hr growth cycle degrading host macromolecules into their monomeric units. For example, host DNA (12, 17) and RNA (6) are degraded into intermediate sized fragments (approx. $1x10^5$ daltons) and soluble nucleotides. These processes are regulated such that over 90% of the host nucleic acids are retained within the bdelloplast. This insures that these materials will remain available for bdellovibrio biosynthetic needs. The fact that DNA degrading activities can be blocked by addition of chloramphenicol, and that bdellovibrios can grow on heat killed host cells, has led to the conclusion that these degradative activities are catalyzed by enzymes of bdellovibrio origin.

The next stage of the cycle, which begins after the first hour, is the period of bdellovibrio growth. Growth is detectable through an increase in the respiration rate (5) and the incorporation of host derived nucleotides into bdellovibrio DNA (12) and RNA (6). In order to generate the energy for growth, the bdellovibrio respires acetate (8, 11), ribose moieties (7) and certain amino acids (8); all of which are derived from the host. The energy obtained from these compounds drives the biosynthesis of bdellovibrio macromolecules. Besides DNA and RNA, host derived monomers are also used for the synthesis of proteins (28) and lipids (11). This pattern, however, is not absolute. The bdellovibrios do have the ability to alter, as well as synthesize *de novo*, a number of cellular components. It is known that host peptidoglycan components are not used for the synthesis of the corresponding bdellovibrio polymer (27). It has also been shown that some bdellovibrio DNA is synthesized from host RNA components (6, 17), and that some bdellovibrio fatty acids are produced by alteration of host fatty acids or synthesized *de novo* from acetate (11).

As the bdellovibrio synthesizes new cell material, it elongates into a coiled filament. At the end of the growth phase this filament fragments into 3-5 individual cells. A terminal lytic activity is produced at the very end of growth (27) that causes dissolution of the bdelloplast wall and release of the progeny bdellovibrio.

In addition to the normal bi-phasic *Bdellovibrio* life cycle just described , some strains can enter a resting stage under certain environmental conditions. For example, *B. bacteriovorus* strain W form resting structures called bdellocysts, in response to changes in growth conditions (29). After penetration and bdelloplast formation, bdellocyst formation can be initiated by transferring strain W cultures from growth medium to a phosphate buffer. Bdellocysts remain in the bdelloplast until germination conditions (addition of NH_4^+) are created (30). Several marine bdellovibrio isolates have also been shown to enter a stable resting stage while remaining in the bdelloplast (19). Return to vegetative growth for these strains occurs after addition of yeast extract

to the growth medium. Structurally, the two types of resting stages differ in that the bdellocysts formed by strain W contain an additional outer wall layer. It has been suggested (19) that the formation of a resting stage by marine bdellovibrios is a survival strategy that occurs in response to nutrient-poor, and therefore host-poor, conditions. The capacity to enter a resting stage may be common among bdellovibrios, even though the proper *in vitro* conditions for its occurence in other strains have not been established.

Intraperiplasmic growth is a highly efficient process. Approximately 50% of the host cell carbon content is converted into bdellovibrio cell material. In addition, Y_{ATP} values calculated for intraperiplasmic growth (14) indicate that relatively little energy is required for the synthesis of bdellovibrio cell material. The Y_{ATP} value (grams dry weight of cell material formed per mole ATP produced) of 19-26 obtained in these experiments compares very favorably to the generally accepted value of about 10 for heterotrophic growth in rich media, and approaches the theoretical maximum of about 30 calculated for an organism expending energy only for the polymerization of monomeric units into cell polymers (14). This impressive efficiency of ATP utilization has been attributed to a combination of: the conservation of phosphate bonds in host nucleic acids (6, 15,) and phospholipids (11); a sequestered, complete nutrient supply (14); and the close coupling of energy generation and utilization (14).

Regulation of *Bdellovibrio* growth and development

The "switch" between the search and growth phases of the *Bdellovibrio* life cycle is stringently controlled. Search phase bdellovibrios cannot grow in complex commercial media (13, 21, 23), nor do they synthesize DNA in the absence of host cells (35). Further, growth phase bdellovbrios immediately differentiate into search phase cells

after premature release from the bdelloplast (18), even if they are suspended in rich growth medium. Thus, bdellovibrios appear to require specific, continuous cues provided by the host cell to remain in the growth phase.

Understanding the mechanism that regulates the alternation between search and growth phase is fundamental to our knowledge of *Bdellovibrio* growth and development. This issue has been addressed in a number of physiological studies. Several groups have reported that high concentrations of host derived, cell-free extracts (1-6 mg protein per ml) can support the axenic growth of bdellovibrios (3, 9, 13), while media containing various combinations of rich commercial media cannot (13, 21, 23). The active component(s) in the extract is heat stable (100^{o}C) and pronase sensitive (3, 9). Further identification of the active component(s) has been unsuccesful. Similarly, a factor involved in *Bdellovibrio* cell division, derived either from the host (4) or the parasite (2) has been suggested, but not identified. Although these approaches retain the potential to further our understanding of bdellovibrio host-dependence, to date they have revealed few specifics.

The control of the *Bdellovibrio* life-cycle has also been studied through the isolation of spontaneous mutants that are capable of growth in the absence of host cells or host extracts. These mutants, termed host-independent (H-I), have been isolated from the three recognized *Bdellovibrio* species, *B. bacteriovorus* (10, 33), *B. stolpii* (1) and *B. starrii* (20, 22). H-I mutants are normally grown on complex bacteriological media containing various mixtures of peptone, yeast extract and nutrient broth. Under these conditions their growth and development is very similar to that of the wild-type grown intraperiplasmically (1, 20). Most H-I isolates retain limited intraperiplasmic (IP) growth capabilities, and form plaques that are much smaller and more turbid than wild-type plaques. After repeated subculture, variants often arise that have lost all IP growth capabilities, presumably through the accumulation of additional mutation(s) (20, 33).

H-I mutants occur at a frequency of 10^{-6} - 10^{-7}, which suggests that they arise from a single mutational event (10, 20, 33, this work). The lack of systems for genetic analysis in *Bdellovibrio* has prevented the identification of such mutations, and thus it is unknown whether the H-I phenotype results from mutation at one or multiple loci. The identification and characterization of H-I mutations could potentially reveal how a single mutation enables a normally host-dependent *Bdellovibrio* to switch back and forth between search and growth phase in the complete absence of normal host cell cues. Two possibilities are: 1) activation of new metabolic capabilities or 2) inactivation of normal regulatory activities associated with host-dependence. Ultimately, defining the basis for H-I growth may, in turn, enhance our understanding of *Bdellovibrio* host-dependence.

Amongst procaryotic obligate intracellular parasites, the *Bdellovibrio*-host system is the only case where anything is known about the genetic basis for host-dependence. Even in the most extensively studied genera, *Chlamydia*, *Coxiella* and *Rickettsia*, no mutants capable of axenic growth have never been isolated and there is a complete lack of systems for genetic manipulation (T. Hackstedt, Rocky Mountain Lab, NIH, personal communication). In light of this, understanding the phenomenon of H-I growth in *Bdellovibrio* is significant, for it could potentially result in the first description of the genetic basis for obligate intracellular parasitism.

The focus of this Dissertation has been to deepen our understanding of bdellovibrio host dependence through the genetic analysis of H-I mutants. Chapter 1 describes the development of a conjugation system for *B. bacteriovorus* 109J, and the use of this system to identify a 5.6 kb DNA fragment from wild-type *B. bacteriovorus* 109J that significantly improves the IP growth of an H-I mutant, BB5. Chapter 2 demonstrates that BB5 and two other H-I mutants contain a mutation within the 5.6 kb fragment. DNA sequence analysis precisely located the mutation in each H-I mutant and defined a locus designated *hit* (host-interaction).

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Chapter 1

Identification of wild-type *Bdellovibrio bacteriovorus* sequences that enhance the plaque forming ability of a spontaneous host-independent mutant

SUMMARY

Bdellovibrio bacteriovorus is an obligate intraperiplasmic parasite of other gram-negative bacteria. Spontaneous mutants of *Bdellovibrio* that can be cultured in the absence of host cells, called host-independent (H-I), occur at a frequency of 10^{-6} - 10^{-7}. Most H-I strains display diminished intraperiplasmic growth capabilities and form plaques that are smaller and more turbid than those formed by wild-type strains on lawns of host cells. In order to begin a genetic analysis of H-I growth, a system for the conjugal transfer of broad host range cloning vectors from *E. coli* into *B. bacteriovorus* 109J was developed. IncQ type plasmids were capable of autonomous replication in *B. bacteriovorus* 109J. IncP derivatives did not replicate in *Bdellovibrio*, but could be maintained via integrational recombination through cloned *Bdellovibrio* sequences. A wild-type *B. bacteriovorus* 109J genomic library was constructed and transferred into the host-independent (H-I) mutant BB5. These experiments resulted in the identification of a 5.6 kb *Bam*HI fragment of wild-type DNA that significantly enhanced the plaque forming ability of BB5.

INTRODUCTION

Bdellovibrio bacteriovorus is an obligate intraperiplasmic parasite of other gram-negative bacteria. Its bi-phasic life cycle revolves around the availability of suitable host cells (reviewed in 22). After contact with a host cell, the bdellovibrio penetrates the host outer envelope and takes up residence within the host periplasm. At some point during the early stages of this interaction, the bdellovibrio undergoes a shift from search phase to growth phase, initiating a complex, temporally regulated series of activities required for growth in this unique environment. At the completion of growth, the progeny return to search phase and lyse the host, causing their release back into nature.

Certain spontaneous *Bdellovibrio* mutations obviate the host requirement, giving rise to mutants that can be cultured in complex bacteriological media (3, 8, 18, 19, 23). Such host-independent (H-I) mutants complete the transition from search phase to growth phase and back again without the normal signals associated with the intraperiplasmic niche. Upon initial isolation, the vast majority of these mutants retain limited intraperiplasmic (IP) growth capabilities, and are termed facultative. When plated on lawns of host cells, facultative H-I mutants form plaques that are smaller and more turbid than plaques formed by wild-type bdellovibrios (3, 19, 23). H-I mutants generally mimic wild-type development when cultured on various mixtures of yeast extract, peptone and nutrient broth. It has been suggested that the H-I phenotype results from a single mutational event, since H-I mutants occur at a frequency of 10^{-6} - 10^{-7} (8, 18, 23, this work). Additional genetic characterization of H-I mutants has been hindered by the absence of systems for the genetic manipulation of bdellovibrios.

The long term goal of this research is to better understand how *Bdellovibrio* regulates the switch between search phase and growth phase, and back again. H-I mutants of *B. bacteriovorus* 109J, such as BB5, have lost the tight control over growth

initiation. Mutation to host-independence may, therefore, affect some fundamental aspect of how bdellovibrios sense the presence of host and control their life cycle. For this reason, I have chosen to investigate the genetic basis for H-I growth in BB5. Towards this end, I have developed a system for the conjugal transfer of broad host range plasmids into wild-type *Bdellovibrio bacteriovorus* 109J and its H-I derivatives. Using this system, I have identified wild-type *B. bacteriovorus* 109J sequences that enhance the plaquing ability of an H-I mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. *B. bacteriovorus* 109J and *E. coli* ML35 were obtained from S. C. Rittenberg. All bdellovibrio strains were single plaque or single colony purified and stored in 15% glycerol at -80°C.

Media and growth conditions. All *E. coli* cultures were grown at 37°C in LB medium (11). When appropriate, *E. coli* cultures contained antibiotics at the following concentrations: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 20 μg/ml; kanamycin sulfate (Km), 25 μg/ml; rifampicin (Rf), 100 μg/ml; streptomycin sulfate (Sm), 50 μg/ml, and tetracycline (Tc), 12 μg/ml.

Intraperiplasmic (IP) cultures of *B. bacteriovorus* 109J and its derivatives were grown in dilute nutrient broth (DNB) at 30°C, using *E. coli* ML35 as substrate. DNB consisted of 1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.8 g nutrient broth (Difco) per liter. Host cells were prepared by washing overnight cultures of ML35 once in an equal volume of DNB. Liquid IP cultures were set up by adding approximately 10^9 host cells and 10^7 bdellovibrios per ml, using single plaques or overnight cultures as the bdellovibrio inoculum. These cultures routinely lysed in 1 day. IP cultures were

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
B. bacteriovorus		
109J	wild-type	(14)
109J.1	Sm^r derivative of 109J	This study
109J.2	Rf^r derivative of 109J.1	This study
BB5	H-I derivative of 109J.2	This study
E. coli		
ML35	B *lacI lacY*	(15)
SR-1	Sm^r Rf^r derivative of ML35	This study
DH5	F^- *endA1 recA1 hsdR17*($r_K^- m_K^+$) *deoR thi-1 supE44* $^-$ *gyrA96 relA1*	(7)
SM10	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21 recA* Mu_c^+ RP4-2Tc::Mu, Km^r	(20)
Plasmids		
pKC7	ColE1 Ap^r Km^r	(13)
pBR328	ColE1 Ap^r Cm^r Tc^r	(21)
pRK2013	ColE1 Km^r *tra*(RK2)	(5)
pSUP204	IncQ Ap^r Cm^r Tc^r	(12)
pSUP304.1	IncQ Ap^r Km^r	(12)
pMMB33	IncQ Km^r *cos*( )	(6)
pRK290	IncP Tc^r	(4)
pVK100	IncP Tc^r Km^r	(9)
pVK102	IncP Tc^r Km^r	(9)
pVKα-1	Derivative of pVK100 containing 445 bp *Hae*II fragment from pUC19	(Chapter 2)
pTC3	23.5 kb fragment of *B. bacteriovorus* 109J DNA in *Eco*RI site of pVK100	This study
pTC5	4.9 kb fragment of *B. bacteriovorus* 109J DNA in *Eco*RI site of pVK100	This study
pTC6	20.5 kb fragment of *B. bacteriovorus* 109J DNA in *Eco*RI site of pVK100	This study
pTC7	19.5 kb fragment of *B. bacteriovorus* 109J DNA in *Eco*RI site of pVK100	This study
pTC8	5.6 kb *Bam*HI fragment from pTC7 in *Bam*HI site of pMMB33	This study
pTC12	5.6 kb *Bam*HI fragment from pTC7 in *Bgl*II site of pVK102	This study
pTC50	0.96 kb *Eco*RI-*Xba*I fragment from pTC12 in *Bam*HI site of pVKα-1	(Chapter 2)

plated for plaque development by adding 0.1 ml of the appropriate bdellovibrio dilution and 10^{10} washed host cells (in 0.3 ml) to 3 ml overlay (DNB plus 0.7% agar held at 50°C), and immediately spread on DNB plates that contained 1.5% agar. Under these conditions plaques became visible after 3-4 days. Rf^r and Sm^r bdellovibrios were grown on *E. coli* SR-1, in the presence of 100 μg/ml Rf or 50 μg/ml Sm. Plasmid containing bdellovibrios were grown on *E. coli* ML35 carrying pKC7 or pBR328, in the presence of 35 μg/ml Km or 10 μg/ml Cm, respectively.

H-I bdellovibrio cultures were grown at 30°C in PYE medium (18) that contained 10 g peptone and 3 g yeast extract per liter. PYE plates were solidified with 1.5% agar. When appropriate, antibiotics were used at the same concentrations as in I-P cultures.

Isolation and characterization of H-I mutant BB5. Spontaneous H-I mutants of *B. bacteriovorus* 109J were obtained at a frequency of 10^{-6} - 10^{-7} using the method of Seidler and Starr (18). Selection for H-I growth yielded yellow CFUs that varied from 0.2 mm - 3.0 mm in diameter. The "class" of spontaneous H-I mutants that formed medium and large colonies could generally be sub-cultured on solid or liquid PYE medium, whereas the small and tiny colony "class" could occasionally be subcultured if several were pooled together to form a large inoculum. A well isolated large colony isolate, BB5, was selected for further characterization. BB5 formed circular, smooth edged colonies that were 1-2 mm in diameter after 7 days incubation on PYE plates at 30°C. BB5 also formed small plaques when plated in overlay lawns of host cells on DNB medium. These plaques were smaller and more turbid than plaques formed by wild-type *B. bacteriovorus* 109J (see Results). Total PFUs formed by BB5 were generally 10-100% the total number of CFUs.

Chemicals and reagents. Complex medium components were purchased from Difco. Restriction endonuclases and T4 DNA ligase were purchased from New England Biolabs. [α-^{32}P]dCTP (800 Ci/mM) was purchased from DuPont/New England Nuclear.

Matings. Individual matings were conducted on 3 cm sq pieces of nitrocellulose (Schleicher and Schuell) that were incubated on PYE plates. The nitrocellulose was autoclaved in water, placed on PYE plates and allowed to dry (30 min at RT). Wild-type *Bdellovibrio* recipients were prepared from freshly lysed IP cultures. Such cultures were concentrated 10-fold by centrifugation, and 0.1 ml of the suspension was spread on a nitrocellulose filter and allowed to dry (30 min at RT). H-I mutant recipients were prepared by placing 0.1 ml of an overnight culture on a nitrocellulose filter, letting it dry (30 min at RT) and then incubating the filter overnight at 30^{o}C on a PYE plate. Donor *E. coli* cultures that had been washed once in DNB and concentrated 10 fold were spread (0.1 ml) on top of the recipients. After 16-24 hrs incubation at 30^{o}C, individual matings (nitrocellulose filters) were transferred to 2 ml DNB and vortexed vigorously, followed by serial dilution and plating for PFUs and CFUs. When plating for axenic growth on PYE plates, Sm (50 μg/ml) was included in the medium to select against growth of the donor. All bdellovibrio recipient cultures were started from -80^{o}C stocks. Donor strains were either *E. coli* SM10 or *E. coli* DH5. In the case of SM10, functions required for the conjugal transfer of IncQ and IncP type plasmids are provided by an IncP plasmid that is integrated into the SM10 genome. When DH5 was used as donor, the same transfer functions were provided by the helper plasmid pRK2013, which is a ColE1 derivative and cannot replicate in *Bdellovibrio* (T. Cotter, unpublished observation). When DH5 was the donor, overnight cultures of DH5 containing the target plasmid and DH5(pRK2013) were mixed in equal volumes and treated as described above.

DNA manipulations, Southern analysis and library construction. Most DNA purification and recombinant DNA methods were standard (16). Bdellovibrio genomic DNAs were purified by a CTAB based extraction procedure (1).

For Southern analysis, bdellovibrio genomic DNA was digested with the appropriate restriction enzymes, fractionated by electrophoresis in 0.7% agarose gels and transferred to Nytran membranes (Schleicher and Schuell) using the capillary method. Prior to hybridization, membranes were prewashed in 0.1X SSPE (1X: 0.18 M NaCl; 1mM EDTA; 10 mM $NaPO_4$, pH=7.7) and 0.5% SDS at 65°C. Prewashed membranes were then prehybridized for 1 hr at 68°C in hybridization fluid that contained 6X SSPE, 0.5% SDS and 0.25% nonfat dry milk (Sanalac). Radiolabeled probe was then added and allowed to hybridize overnight at 68°C. All post-hybridization washes contained 0.5% SDS and were done in the following order: twice in 2X SSPE at RT; twice in 0.1X SSPE at RT and three times in 0.1X SSPE at 68°C. Radiolabeled probes (approximately 10^7 dpm/μg DNA) were produced by nick translation (kit obtained from BRL) or random priming (16).

The *B. bacteriovorus* 109J genomic cosmid library TVL-1 was constructed according to the method described by Ausubel et al. (1). Genomic DNA was partially digested with *Eco*RI, and size fractionated in 0.5% agarose gels; DNA fragments were electroeluted from the gel and purified with elutip-d columns (Schleicher and Schuell). The size fractionated DNA (20-30 kb) was ligated into the *Eco*RI site of pVK100 (9), and the ligation products packaged with commercial extracts (Promega) according to supplier specifications. Packaged cosmids were transduced into DH5 and stored at -80°C.

RESULTS

Conjugal transfer of RSF1010 and RK2 derivatives into *B. bacteriovorus*. Previous studies have shown that RK2 transfer functions supplied *in trans* can provide the means for conjugal transfer of IncQ (RSF1010) and IncP (RK2) derived plasmids from one gram-negative species to another (4, 12). We attempted to transfer both types of plasmids into *B. bacteriovorus* 109J by conjugation, using *E. coli* as the donor (see Materials and Methods). Conjugal transfer of the RSF1010 derivatives pSUP204, pSUP304.1 and pMMB33 produced antibiotic resistant recipients of host-dependent (H-D) and host-independent (H-I) *Bdellovibrio* strains (Table 2). Matings conducted in the absence of RK2 transfer functions did not yield antibiotic resistant *Bdellovibrio* recipients, indicating that plasmid transfer was conjugal in nature (data not shown). Km^r (20-40 μg/ml) and Cm^r (5-10 μg/ml) were effective in selecting for the transfer of RSF1010 derivatives, while Tc^r (2-25 μg/ml) and Ap^r (5-50 μg/ml) resistance were not.

Two lines of evidence indicated that RSF1010 derivatives were maintained in *B. bacteriovorus* 109J by autonomous replication. Southern analysis of total DNA isolated from BB5(pMMB33) revealed a single hybridizing band of 13.8 kb, representing linear pMMB33 (Figure 1A). In addition, BB5(pMMB33) total DNA was used to transform *E. coli* DH5 to antibiotic resistance, and the transformants were shown to contain pMMB33 (data not shown).

All attempts to conjugally transfer the RK2 derivatives pRK290 and pVK100 into H-D and H-I strains of *B. bacteriovorus* failed to yield antibiotic resistant recipients (Table 2). Since RSF1010 plasmids were mobilized into bdellovibrio by RK2 transfer functions, it was possible that RK2 plasmids were also transferred but could not replicate. If true, insertion of *B. bacteriovorus* 109J sequences into a RK2 derivative could potentially allow such constructs to be maintained in bdellovibrio after integration

Table 2. Conjugal transfer of plasmids into *B. bacteriovorus*[a]

Plasmid	Transfer frequency[b]	Useful antibiotic selections
IncQ		
pSUP204	$1x10^{-3}$	Cm^r, Km^r
pSUP304.1	$1x10^{-3}$	Km^r
pMMB33	$1x10^{-3}$	Km^r
IncP		
pRK290	0	--
pVK100	0	--
pTC3	$1x10^{-4}$	Km^r

[a] Data apply to matings involving H-D or H-I strains as recipient, and either SM10 or DH5(pRK2013) as donor.

[b] Transfer frequency expressed as number of antibiotic recipients per total recipients

Figure 1. Southern analysis of plasmid containing *B. bacteriovorus*. (A) *Bam*HI digests of total DNA isolated from BB5 (lane 1) and BB5(pMMB33) (lane 2) probed with nick translated pMMB33. (B) *Bam*HI digests of total DNA isolated from BB5 (lane 1) and BB5(pTC50) (lane 2) probed with the random prime labelled 5.6 kb *Bam*HI insert from pTC12.

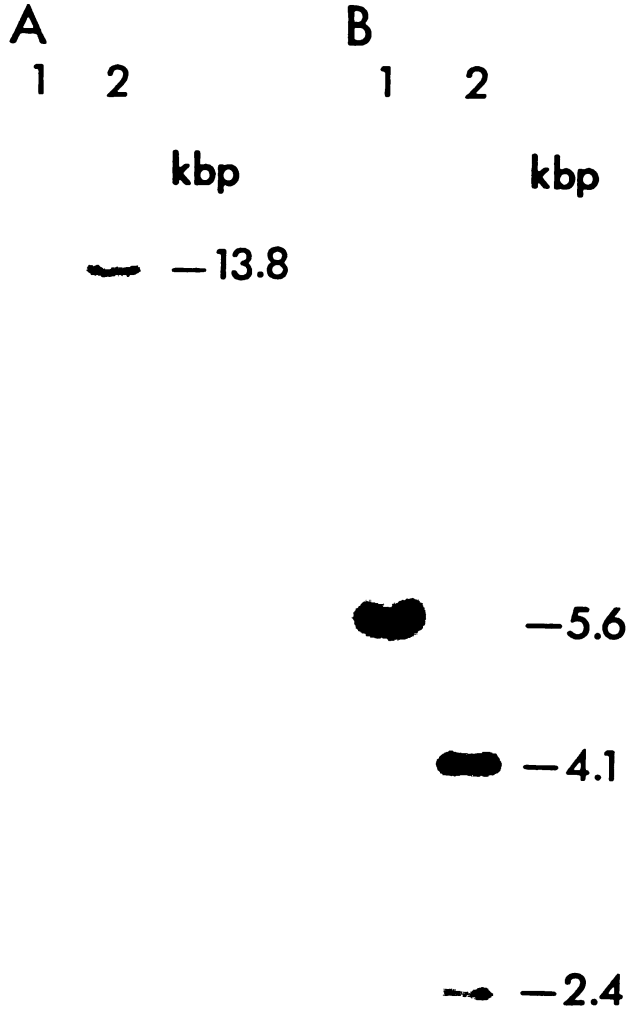

Figure 1.

by homologous recombination. Indeed, in contrast to the cloning vector alone, the conjugal transfer of pTC3, an RK2 derivative (pVK100) containing a random 23.5 kb fragment of *B. bacteriovorus* 109J DNA, produced kanamycin resistant recipients of both wild-type and H-I mutant derivatives of *B. bacteriovorus* 109J (Table 2). The transfer frequency of pTC3 was approximately 10-fold less than that of RSF1010 derivatives, presumably due to the requirement for recombination.

The integration of RK2 based constructs into the bdellovibrio genome was demonstrated by Southern analysis of total DNA obtained from BB5(pTC50) (Figure 1B). pTC50 contains a 0.96 kb *Eco*RI-*Xba*I fragment of *B. bacteriovorus* 109J DNA derived from the 5.6 kb bdellovibrio DNA insert in pTC12 (described below). The natural *Xba*I and *Eco*RI termini of the 0.96 kb fragment were replaced with *Bam*HI termini prior to cloning into the *Bam*HI site of pVKα-1 (Chapter 2). Based on the physical characterization of this region (Chapter 2), integration of pTC50 into the BB5 genome by homologous recombination should transform the 5.6 kb *Bam*HI fragment into two *Bam*HI fragments of 4.1 and 2.4 kb. This prediction proved to be true (Figure 1B). Total DNA from BB5 (lane 1) contained the expected single hybridizing band representing the 5.6 kb *Bam*HI fragment, whereas a *Bam*HI digest of BB5(pTC50) contained two smaller fragments of 4.1 and 2.4 kb.

Identification of wild-type sequences that enhance plaquing activity in H-I mutant BB5. The H-I mutant BB5, like other described H-I mutants, retains a diminished capacity for intraperiplasmic (IP) growth, forming small turbid plaques on lawns of host cells (Figure 2). If the mutations that result in the H-I phenotype are due to gene inactivation, then it might be possible to identify the affected region by transferring a wild-type genomic DNA library into BB5 and screening the exconjugants for plaquing ability that is significantly enhanced over that displayed by BB5.

Figure 2. Enhanced-plaque phenotype of BB5(pTC12). Plaques formed by (A) 109J.2(pTC3) (wild-type), (B) BB5(pTC3) (H-I) and (C) BB5(pTC12) are compared. pTC3 does not affect the plaque phenotype of wild-type or mutant strains and is used here as a control to confer Km^r.

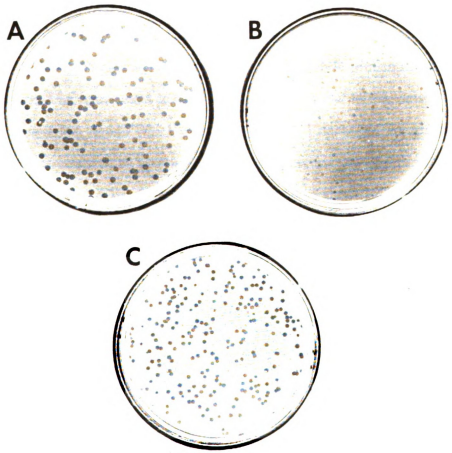

Figure 2.

I first attempted to construct a wild-type genomic DNA library in pMMB33 (6), which could autonomously replicate in *B. bacteriovorus* 109J. Initially this construction appeared to be succesful, but continued propagation of several individual clones in *E. coli* indicated that pMMB33 could not stably maintain large *Bdellovibrio* DNA inserts (25-35 kbp). A stable cosmid library of *B. bacteriovorus* 109J genomic DNA, however, was constructed in pVK100 (see Materials and Methods). Given that the packaging process should restrict insert size to between 22 and 28 kbp and that the *B. bacteriovorus* 109J genome is 2.0×10^6 bp (10), I calculated according to Clark and Carbon (2) that 366 clones would be required to assure 99% probability that any given sequence would be represented in the library. Based on that figure, several genomic libraries were made, one of which was TVL-1. TVL-1 contained approximately 700 clones, and was constructed by combining six independent sub-libraries (VL-1, VL-2, VL-3, VL-4, VL-5 and VL-6), each of which contained about 100 clones.

VL-1 through VL-6 were individually mated into BB5, and the recipients screened for improved plaquing ability. Sub-libraries VL-3 and VL-4, as well as pTC3 (which served as the negative control), did not confer an enhanced plaquing phenotype upon any BB5 recipients. Sub-libraries VL-1, VL-2, VL-5 and VL-6, however, gave rise to larger, clearer PFUs that comprised 1-2% of the total Km^r recipients.

If the enhancement of BB5 plaquing activity resulted from homologous recombination of wild-type sequences into the recipient genome at the site of the H-I mutation, then identical or related cosmids should have been present in the enhanced plaque recombinants. RFLP analysis was conducted to determine if this was the case. Total DNAs were digested with *Hind*III, an enzyme that cuts once within pVK100, and subjected to Southern analysis using pVK100 as probe (Figure 3). Each digest would be expected to contain 2 bands that hybridized to the cloning vector, both of which which would have extended in opposite directions from the *Hind*III site within pVK100 to *Hind*III sites in the adjacent bdellovibrio DNA (Figure 3A). When 18 random

Figure 3. RFLP analysis of cosmid containing exconjugants. (A) Schematic diagram showing co-integration of a cosmid into the recipient *Bdellovibrio* genome, and the 2 hypothetical *Hin*dIII fragments that hybridize to the pVK100 probe. Restriction site: H, *Hin*dIII. Hypothetical cloned *Bdellovibrio* DNA represented by the solid box. pVK100 represented by the hatched box. The thin line represents the recipient genome. (B) *Hin*dIII digests of total DNA isolated from 18 random BB5 recipients probed with nick translated pVK100. Individual Km^r isolates were obtained after mating with sub-libraries VL-1 (lanes 1-9) and VL-2 (lanes 10-18). (C) *Hin*dIII digests of total DNA isolated from 18 enhanced-plaque BB5 recipients probed with nick translated pVK100. Individual Km^r isolates were obtained after mating with sub-libraries VL-1 (lanes 1-6), VL-2 (lanes 7-12) and VL-5 (lanes 13-18).

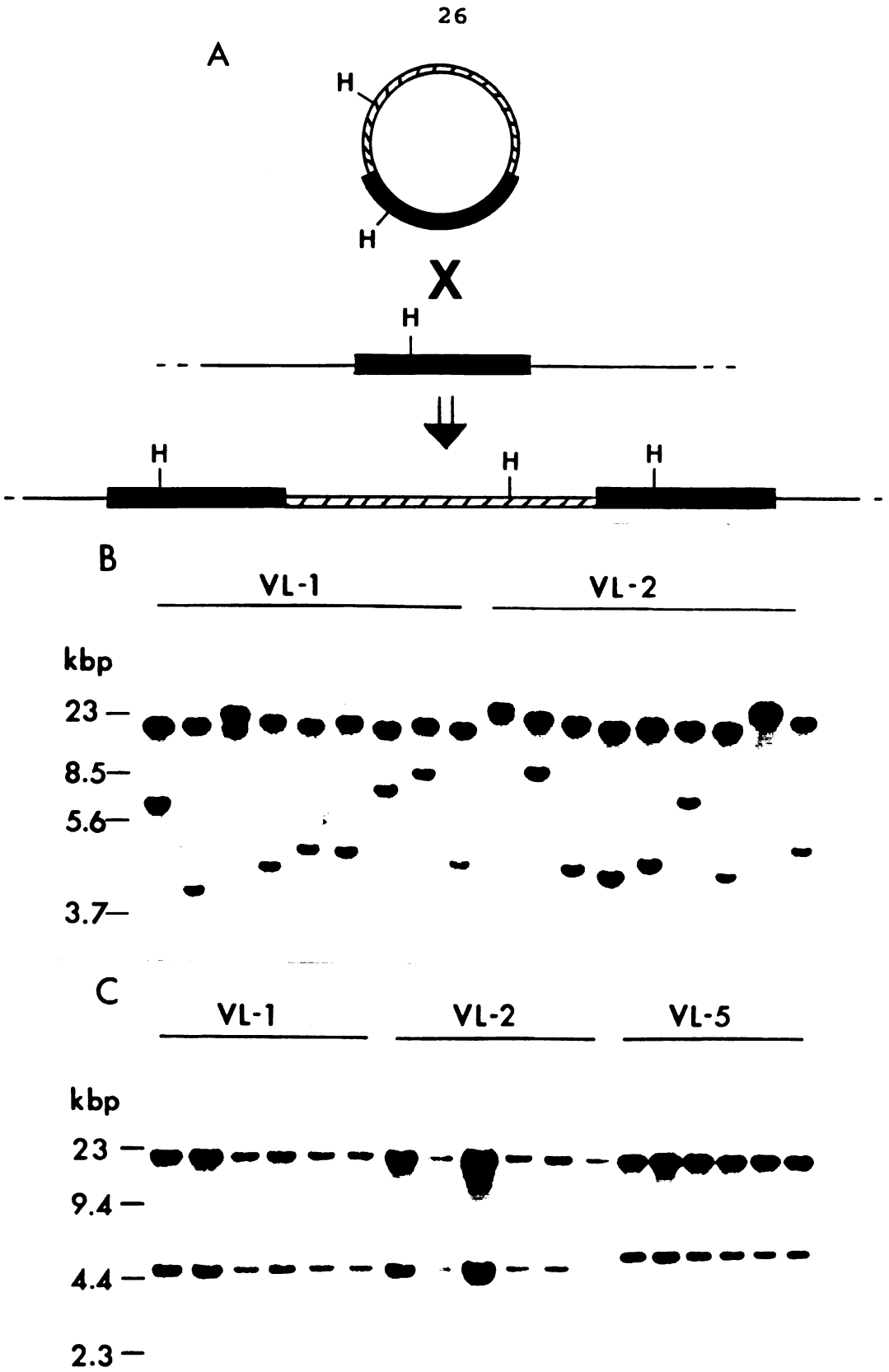

Figure 3.

recipients were analyzed in this way, 17 distinct patterns were observed (Figure 3B). These results contrasted with those obtained from RFLP analysis of enhanced-plaque exconjugants from VL-1, VL-2 and VL-5. All of the BB5 recombinants that contained cosmids from VL-1 (9 individuals) and VL-2 (8 individuals) displayed the same RFLP pattern. A second, distinct pattern was seen in 10 isolates that contained cosmids from VL-5. These two RFLP patterns are seen in Figure 3C, which shows 6 individuals each from VL-1, VL-2 and VL-5. These data indicated that specific regions of the *B. bacteriovorus* genome were involved in conferring the enhanced-plaque phenotype upon BB5.

Further characterization of wild-type sequences responsible for plaque enhancement in BB5 required the isolation of an entire cosmid from an enhanced-plaque BB5 exconjugant. This was accomplished in several steps. From an enhanced-plaque BB5 exconjugant that showed the predominant RFLP pattern (lane 5, Figure 3C), total DNA was isolated and digested with *Bam*HI. Since *Bam*HI does not cut within pVK100, the integrated vector was released with flanking bdellovibrio sequences attached to each end. This linear, vector-containing *Bam*HI fragment was then circularized in a dilute ligation and transformed into *E. coli* DH5. A single plasmid, pTC5, was identified that contained pVK100 plus 2 flanking *Eco*RI-*Bam*HI fragments of 2.3 and 2.6 kb. The 4.9 kb *Eco*RI insert from pTC5 was purified and used to probe colony lifts of VL-1. Two cosmids that hybridized to the pTC5 insert, pTC6 and pTC7, were isolated and found to contain overlapping inserts of 20.5 kb and 19.5 kb, respectively (Figure 4).

Cosmids pTC6 and pTC7 were tested for the ability to confer the enhanced-plaque phenotype upon BB5. Recombinants containing pTC6 had the same plaque phenotype as the negative control, BB5(pTC3), whereas BB5(pTC7) exhibited the enhanced-plaque phenotype. From within pTC7, a 5.6 kb *Bam*HI fragment was identified, carried on pTC12, that also enhanced plaque development in BB5 (Figure 4).

Figure 4. Plasmid constructs containing *B. bacteriovorus* 109J DNA and their ability to enhance plaque formation in H-I mutant BB5. Inserts from plasmid constructs that contain overlapping fragments of *B. bacteriovorus* 109J DNA are shown. Restriction sites: B, *Bam*HI; E, *Eco*RI.

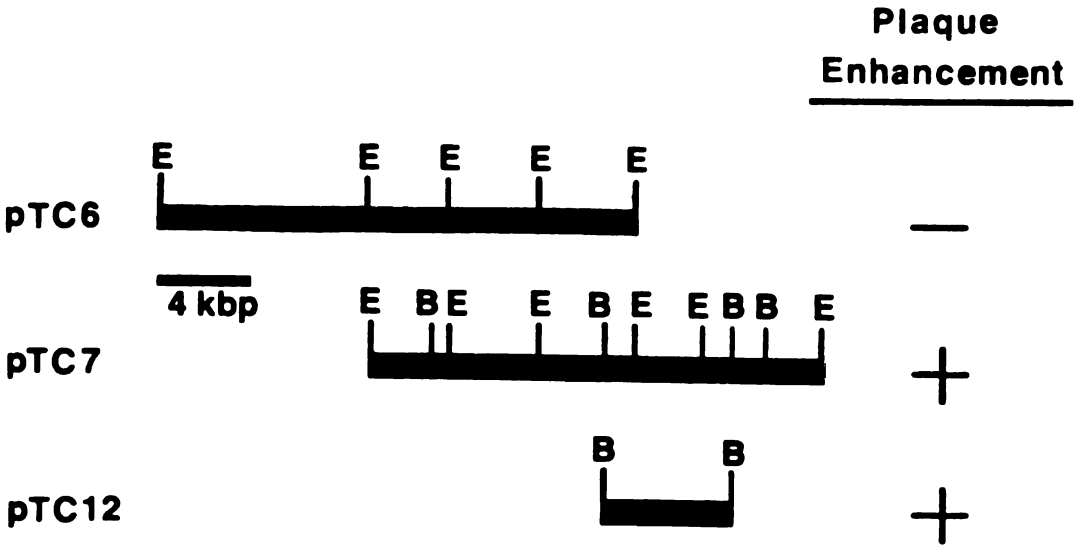
Plaque
Enhancement
E
E
E
E
E
pTC6
—
4 kbp
E
B
E
E
B
E
E
B
B
E
pTC7
+
B
B
pTC12
+

Figure 4.

BB5(pTC12) plaques, pictured in Figure 2, appeared identical to plaques formed by BB5(pTC7). These plaques were very similar to those formed by the wild-type, except for a moderate reduction in diameter.

DISCUSSION

To date, studies on *Bdellovibrio* growth and development have involved biochemical, physiological and observational experimentation. These approaches can now be complemented by genetic analysis. In particular, we have shown that IncQ and IncP plasmids can be conjugally transferred from *E. coli* into *B. bacteriovorus* 109J. IncQ plasmids are maintained in *B. bacteriovorus* 109J by autonomous replication. IncP plasmids cannot replicate in *B. bacteriovorus* 109J, but can be maintained via homologous recombination and integration through cloned *Bdellovibrio* sequences. This system allows for the transfer of genomic libraries, which dramatically expands the potential for genetic analysis in *Bdellovibrio*. Conjugation should also facilitate the delivery of transposons into the bdellovibrio genome. Indeed, preliminary results (T. Cotter, unpublished data) indicate that Tn*5* can be delivered via suicide plasmids, albeit at low frequency (10^{-8}). Presumably, transposon mutagenesis could be further developed into a useful tool for the genetic analysis of *B. bacteriovorus*.

Here I use the *Bdellovibrio* conjugation system to initiate a genetic characterization of *Bdellovibrio* H-I mutants. H-I mutants have lost the stringent control over growth, being able to complete the transition from search phase to growth phase and back again, in the absence of host cells. Understanding the genetic basis for H-I growth may reveal fundamental information concerning the mechanism that regulates the switch between search and growth phases, and provide insight into the nature of host-dependency in *Bdellovibrio*. Towards this end, I have identified a 5.6 kb *Bam*HI fragment of wild-type *B. bacteriovorus* 109J DNA that confers an enhanced-plaque

phenotype upon H-I mutant BB5. The 5.6 kb fragment was isolated from one of the two cosmids identified as conferring an enhanced-plaque phenotype upon BB5 (Figure 3C), leaving open the possibility that the other cosmid contained unrelated sequences from another region of the *Bdellovibrio* genome. Additional Southern analysis (T. Cotter, unpublished results) indicates that the 2 RFLP patterns seen in Figure 3C are derived from related cosmids, suggesting that the 5.6 kb *Bam*HI fragment is the only region of the *B. bacteriovorus* genome that can confer this phenotype. In the following chapter I further characterize the 5.6 kb fragment from wild-type, BB5 and two additional H-I mutants.

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Chapter 2

Identification of a *Bdellovibrio bacteriovorus* locus, *hit* (host-interaction), that contains a mutation in three spontaneous host-independent mutants.

SUMMARY

Previous work (Chapter 1) has identified a 5.6 kb *Bam*HI fragment of wild-type *Bdellovibrio bacteriovorus* 109J DNA that significantly enhances the plaque forming ability of the host-independent (H-I) mutant BB5. Further genetic analysis, described here, has narrowed down the sequences required to confer this phenotype to a 959 bp *Eco*RI-*Xba*I fragment. DNA sequence analysis of this fragment, and the equivalent fragment from BB5, indicates that BB5 contains a single base pair deletion in this region. Two additional H-I mutants, BB3 and BB4, are also shown to contain deletion mutations within the 959 bp *Eco*RI-*Xba*I fragment. Because of its affect on plaque formation in H-I mutants, and the occurrence of mutation within this region in three H-I mutants, this locus is termed *hit*, for host-interaction. Merodiploid recombinants that contained both wild-type and mutant derived *hit* sequences displayed "intermediate" phenotypes. BB5 recombinants that contained wild-type *hit* sequences formed plaques that were very similar to those of the wild-type (enhanced-plaque phenotype), but could still form colonies that were indistinguishable from those formed by BB5. Recombination of mutant *hit* sequences into the wild-type yielded recombinants that formed plaques which were also very similar to wild-type, but

displayed a capacity for "density-dependent" axenic growth. The genetic basis for the intermediate phenotypes is discussed.

INTRODUCTION

Members of the genus *Bdellovibrio* are obligate intraperiplasmic parasites of other gram-negative bacteria. Their unique life-cycle involves the alternation between a free swimming search phase and an intraperiplasmic growth phase (reviewed in 20). While in the search phase, bdellovibrios are highly motile and metabolically active, but do not replicate their DNA. Upon contact with a host cell, the parasite attaches to, and then rapidly penetrates, the host outer envelope and becomes lodged within the periplasmic space. During these early stages of the interaction with host cells, the bdellovibrio undergoes a transition from search phase to growth phase. In the initial stages of the growth cycle, the parasite conducts the partial degradation of many host cell components. These products are then used for biosynthesis and energy generation as the bdellovibrio elongates into a coiled, multicellular filament. At the cessation of growth, the filament divides into individual search phase progeny that are released back into the environment. *In vitro* the entire cycle requires about 3.5 hours to complete.

The control of bdellovibrio growth is strict: all attempts to bypass the host cell requirement with commercial media have been unsuccessful (4, 10, 16). However, several studies have demonstrated that concentrated cellular extracts from hosts and other bacteria can induce wild-type bdellovibrios to enter the growth phase and support the completion of the entire growth cycle (4, 6, 10). No specific active factor has been identified in these extracts, but rudimentary analyses suggest that a heat-stable proteinaceous component may be involved (4, 6). To date, it is unknown how these extracts stimulate *Bdellovibrio* growth.

The requirement for a host cell can also be bypassed genetically. Spontaneous mutants of wild-type "host-dependent" (H-D) *Bdellovibrio* have been isolated that are able to grow in the absence of hosts or host extracts. Such "host-independent" (H-I) mutants have been obtained from each of the three recognized *Bdellovibrio* species, *B. bacteriovorus* (7, 21), *B. stolpii* (2) and *B. starrii* (15, 17). In addition to being able to grow axenically on complex bacteriological media, most H-I strains retain limited intraperiplasmic (IP) growth capabilities. Such mutant strains are termed facultative, forming smaller, more turbid plaques than wild-type strains on lawns of host cells. Variants of facultative H-I strains that are incapable of IP growth have also been isolated (15, 21). The axenic growth and development of H-I mutants mimics that of wild-type strains growing intraperiplasmically.

H-I mutants arise at a frequency of 10^{-6} - 10^{-7}, suggesting that they can result from a single mutational event at one or multiple loci. A detailed characterization of the genetic locus or loci affected in H-I mutants should provide insight into the regulation of the bdellovibrio developmental cycle. Toward this end, we have identified vectors that can be used to transfer DNA into bdellovibrios by conjugation and have used the system to identify a 5.6 *Bam*HI fragment from wild-type *B. bacteriovorus* 109J that greatly enhances the plaquing ability of an H-I mutant (Chapter 1). Here we describe further genetic and molecular characterization of this region from wild-type *B. bacteriovorus* 109J and three independent H-I mutants. The data indicate that each mutant has suffered a mutation within this region of DNA and define a locus, *hit* (host-interaction), that affects the interaction of *Bdellovibrio* with host cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *B. bacteriovorus* 109J and *E. coli* ML35 were

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
B. bacteriovorus		
109J	wild-type	(11)
109J.1	Sm^r derivative of 109J (other paper)	
109J.2	Rf^r derivative of 109J.1	(Chapter 1)
BB3	H-I derivative of 109J.1	This study
BB4	H-I derivative of 109J.1	This study
BB5	H-I derivative of 109J.2	(Chapter 1)
E. coli		
ML35	B *lacI lacY*	(12)
DH5	F^- *endA1 recA1* *hsdR17*($r_K^- m_K^+$) *deoR thi-1* *supE44* *gyrA96 relA1*	(5)
DH5α	F^- φ80d*lacZ* M15 (*lacZYA* *argF*)U169 *endA1 recA1* *hsdR17*($r_K^- m_K^+$) *deoR thi-1* *supE44* *gyrA96 relA1*	Bethesda Research Laboratories
DH5αF'	F' φ80d*lacZ* M15 (*lacZYA* *argF*)U169 *endA1 recA1* *hsdR17*($r_K^- m_K^+$) *deoR thi-1* *supE44* *gyrA96 relA1*	Bethesda Research Laboratories
SM10	*supE44 hsdR thi-1 thr-1 leuB6* *lacY1 tonA21 recA* Mu_c^+ RP4-2Tc::Mu, Km^r	(18)
Plasmids		
pKC7	ColE1 Ap^r Km^r	(9)
pUC18	ColE1 Ap^r	(22)
pUC19	ColE1 Ap^r	(22)
pRK2013	ColE1 Km^r *tra*(RK2)	(3)
pVK100	IncP Tc^r Km^r	(8)
pVK102	IncP Tc^r Km^r	(8)
pVKα-1	pVK100 derivative containing the 445 bp *Hae*II fragment from pUC19 in *Eco*RI site	This study
pTC3	23.5 kb fragment of *B. bacteriovorus* 109J DNA in *Eco*RI site of pVK100	(Chapter 1)
pTC7	19.5 kb fragment of *B. bacteriovorus* 109J DNA in *Eco*RI site of pVK100	(Chapter 1)
pTC12	5.6 kb *Bam*HI fragment from pTC7 in *Bgl*II site of pVK102	(Chapter 1)
pTC16	5.6 kb *Bam*HI fragment of BB3 DNA in *Bam*HI site of pUC18	This study
pTC17	5.6 kb *Bam*HI fragment of BB4 DNA in *Bam*HI site of pUC18	This study

Table 1 (cont'd).

pTC20	5.6 kb *Bam*HI fragment of BB5 DNA in *Bam*HI site of pUC18	This study
pTC28	5.6 kb *Bam*HI fragment from pTC16 in *Bgl*II site of pVK102	This study
pTC30	5.6 kb *Bam*HI fragment from pTC17 in *Bgl*II site of pVK102	This study
pTC32	5.6 kb *Bam*HI fragment from pTC20 in *Bgl*II site of pVK102	This study
pTC35	2.5 kb *Bam*HI-*Xba*I fragment from pTC12 in *Bam*HI-*Xba*I site of pVKα-1	This study
pTC36	3.1 kb *Bam*HI-*Xba*I fragment from pTC12 in *Bam*HI-*Xba*I site of pVKα-1	This study
pTC37	2.8 kb *Eco*RI fragment from pTC12 in *Eco*RI site of pVKα-1	This study
pTC50	0.96 kb *Eco*RI-*Xba*I fragment from pTC12 in *Bam*HI site of pVKα-1	This study
pTC56	0.96 kb *Eco*RI-*Xba*I fragment from pTC32 in *Bam*HI site of pVKα-1	This study

obtained from S. C. Rittenberg. Antibiotic resistant mutants and H-I mutants of *B. bacteriovorus* 109J were isolated as described by Seidler and Starr (15). All bdellovibrio strains were single plaque or single colony purified and stored in 15% glycerol at -80°C. Media and culture conditions used for the propagation of all *E. coli* and *Bdellovibrio* strains was as previously described (Chapter 1).

Chemicals and reagents. Complex medium components were purchased from Difco. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. [α-^{32}P]dCTP (800 Ci/mM) and [α-35S]dATP (500 Ci/mM) were purchased from DuPont/New England Nuclear.

Matings. The conjugal transfer of plasmids from *E. coli* to *B. bacteriovorus* was done as previously described (Chapter 1). All of the genetic experiments described here involved the the conjugal transfer of bdellovibrio DNA containing IncP plasmids, and their integration into the recipient bdellovibrio genome via homologous recombination. All of the recipients were therefore merodiploid for the cloned sequences. Southern blot analysis has previously shown that integration of *Bdellovibrio* DNA containing constructs occurs via homologous recombination between the cloned *Bdellovibrio* insert and the equivalent region of the recipient genome (Chapter 1).

DNA manipulations and construction of pVKα-1. Most DNA purification and recombinant DNA methods were those of Sambrook et al. (13). Bdellovibrio genomic DNAs were purified by a CTAB based extraction procedure (1).

The 5.6 kb *Bam*HI fragment that contains part or all of the *hit* locus was cloned from H-I mutants BB3, BB4 and BB5 by isolating genomic *Bam*HI fragments that ranged from 5.0 to 6.0 kb in size and ligating them into pUC18. *Bam*HI fragments in that size range were isolated by electroelution (13) after agarose gel electrophoresis.

pTC16 (BB3), pTC17 (BB4) and pTC20 (BB5) were identified as containing the correct 5.6 kb *Bam*HI fragments by colony hybridization (13) using pTC12 as probe. To allow for their conjugal transfer into bdellovibrio, the three mutant derived *Bam*HI fragments were subcloned into the *Bgl*II site of pVK102 yielding pTC28 (BB3), pTC30 (BB4) and pTC32 (BB5).

pVKα-1 was constructed by cloning the 445 bp *Hae*II fragment from pUC19 (that contains the polylinker and α-complementation sequences) into the *Eco*RI site of pVK100. Prior to ligation, the overhanging termini of each fragment were removed by treatment with the Klenow fragment. pVKα-1 allows the use of α-complementation for cloning and contains unique *Bam*HI, *Kpn*I, *Pst*I, *Sst*I and *Xba*I restriction sites within the polylinker.

DNA sequencing. DNA sequences were determined by the dideoxy chain-termination method (14) using the Sequenase procedure of United States Biochemical with single stranded M13 DNA as template. Sequence of the wild-type 959 bp *Eco*RI-*Xba*I fragment was obtained from both strands. The equivalent, mutant derived fragments were sequenced in their entirety on one strand, using primers that were designed according to the wild-type sequence. Discrepancies with the wild-type sequence were confirmed by sequencing on the complementary strand. Open reading frame and similarity analyses were performed on a VAX 8650 computer using University of Wisconsin GCG protein and DNA analysis software (version 6.0).

RESULTS

Identification of a mutation in H-I mutant BB5. We previously showed that recombination of pTC12 into the genome of *B. bacteriovorus* BB5 greatly enhances the plaquing ability of this H-I mutant (Chapter 1; Figure 1). A likely explanation of this

Figure 1. pTC32 does not confer an enhanced-plaque phenotype upon BB5. Plaques formed by (A) 109J.2(pTC3) (wild-type), (B) BB5(pTC3) (H-I), (C) BB5(pTC12) and (D) BB5(pTC32) are compared. Although pTC12 and pTC32 contain equivalent 5.6 kb *Bam*HI inserts derived from wild-type and H-I mutant BB5, respectively, pTC32 does not confer enhanced plaquing in BB5. pTC3 does not affect the plaque phenotype of H-D or H-I strains and is used here as a control to confer Km^r.

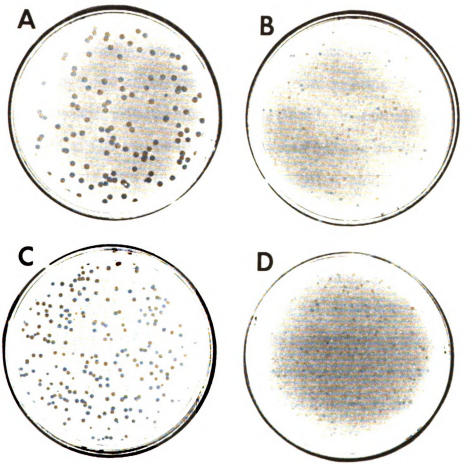

Figure 1.

result was that BB5 contained a mutation within the region of the genome corresponding to the 5.6 kb *Bam*HI fragment cloned in pTC12 and that recombination of the wild-type sequences at this locus resulted in at least a partial correction of the original genetic lesion. To test this hypothesis, we isolated the 5.6 kb *Bam*HI region from BB5 (carried on pTC32) and compared it to that of the wild-type fragment at the physical and genetic levels.

Comparative restriction analysis of wild-type and mutant derived 5.6 kb *Bam*HI fragments did not reveal any obvious deletions or rearrangements. Genetic experiments, however, indicated otherwise. Specifically, in contrast to pTC12, recombination of pTC32 into the BB5 genome did not enhance the plaquing ability of BB5 (Figure 1). To narrow down the site of the mutation, various portions of the 5.6 kb *Bam*HI fragment from pTC12 were subcloned, yielding pTC35, pTC36, pTC37 and pTC50 (Figure 2), and the smaller DNA fragments were tested for whether they could enhance the plaquing ability of BB5. When recombined into BB5, pTC36 had no effect on plaque morphology whereas pTC35, pTC37 and pTC50 mimicked the effects of pTC12 (Figure 2). These data suggested that BB5 had suffered a mutation within the *Eco*RI-*Xba*I fragment cloned in pTC50. This conclusion was confirmed by DNA sequence analysis (Figure 3). The data indicated that the corresponding *Eco*RI-*Xba*I fragments from wild-type *B. bacteriovorus* 109J and its H-I derivative BB5 differed by one base pair; the mutant had a single base pair deletion located 208 bp from the *Xba*I site (marked by the closed circle in Figure 3). As this region of the genome had an effect on *Bdellovibrio* plaquing, the locus was designated *hit* (host-interaction).

The *hit* locus is altered in other spontaneous H-I mutants of *B. bacteriovorus* 109J. The occurence of a mutation within the *hit* locus of BB5 raised the question of whether this locus was commonly affected in H-I mutants derived from *B. bacteriovorus* 109J. I addressed this issue by isolating two additional H-I mutants,

Figure 2. Plasmid constructs containing *B. bacteriovorus* 109J DNA and their ability to enhance plaque formation in H-I mutant BB5. Inserts from plasmid constructs that contain overlapping fragments of *B. bacteriovorus* 109J DNA are shown. Insert sizes are: pTC12, 5.6 kb; pTC35, 2.4 kb;pTC36, 3.2 kb; pTC37, 2.8 kb; pTC50, 0.96 kb. Restriction sites: B, *Bam*HI; E, *Eco*RI; X, *Xba*I.

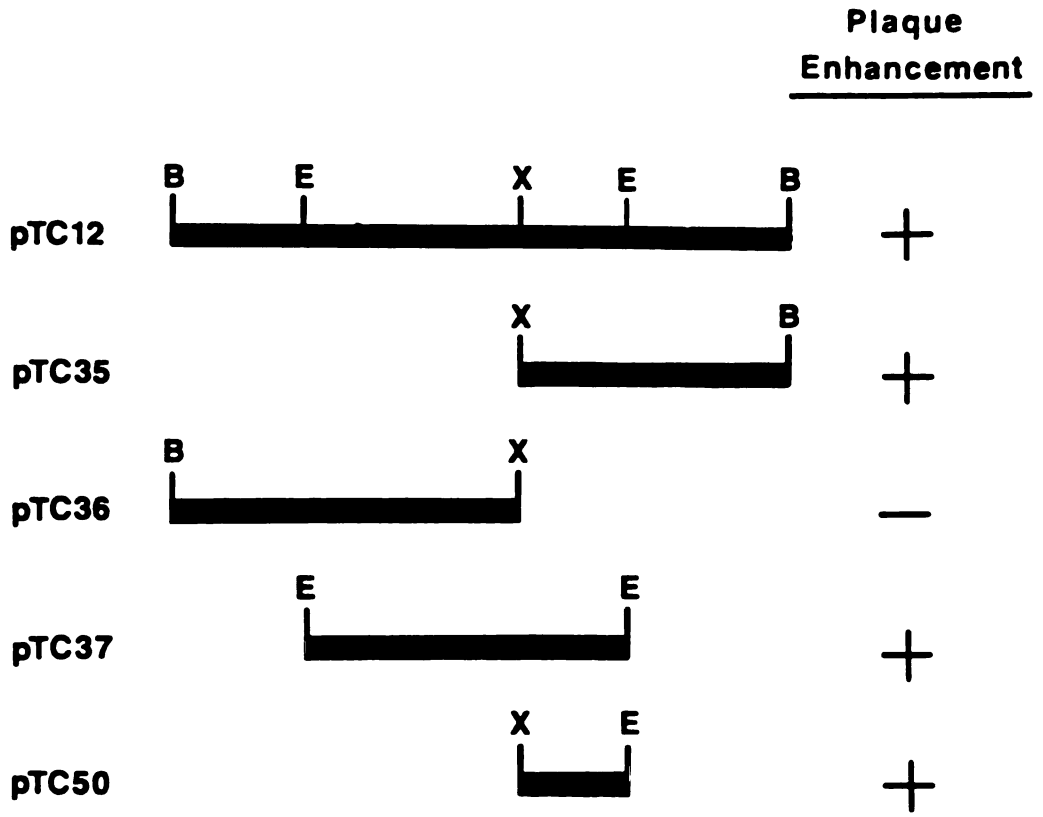
Plaque
Enhancement
B
E
X
E
B
pTC12
+
X
B
pTC35
+
B
X
pTC36
—
E
E
pTC37
+
X
E
pTC50
+

Figure 2

Figure 3. DNA sequence of the 959 bp EcoRI-XbaI fragment from the *hit* locus. Closed triangle, bracket and closed circle symbols indicate locations of deletion mutations in BB3, BB4 and BB5, respectively. Underlining indicates 10 bp direct repeats located at borders of deletion in BB4.

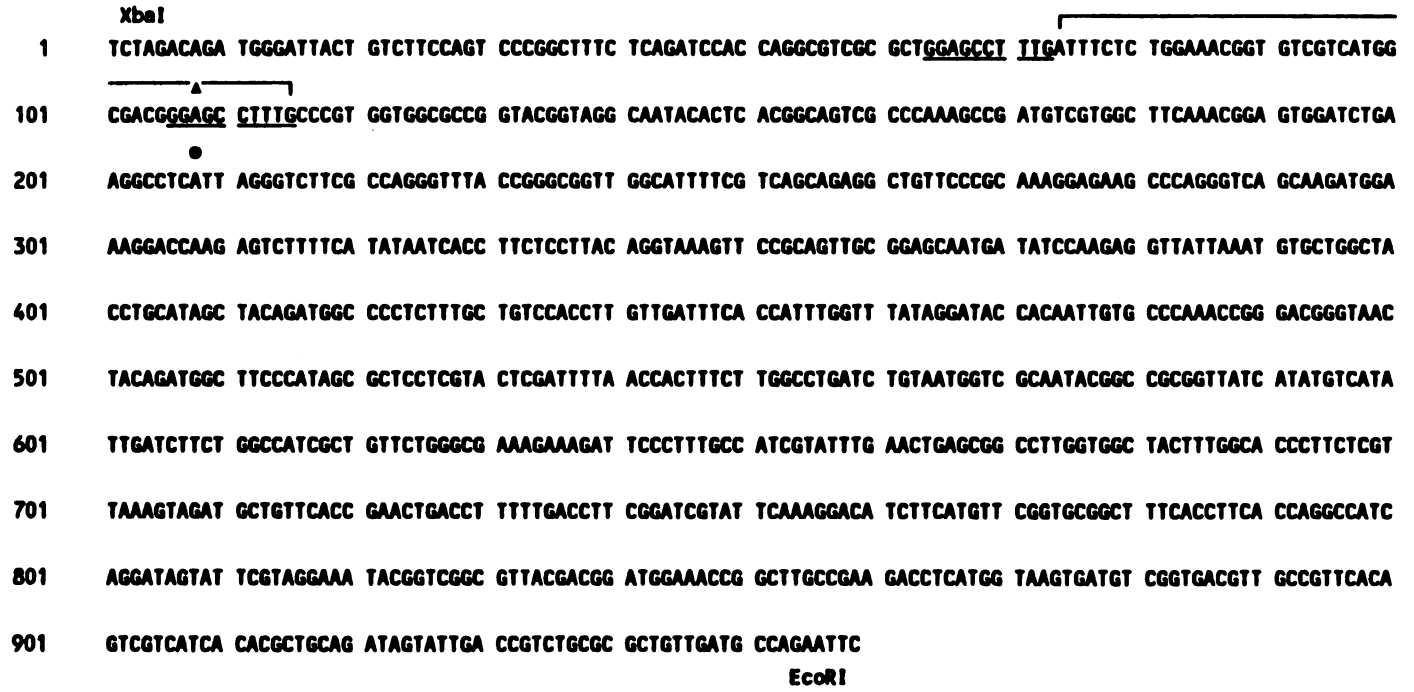
XbaI
1 TCTAGACAGA TGGGATTACT GTCTTCCAGT CCCGGCTTTC TCAGATCCAC CAGGCGTCGC GCTGGAGCCT TTGATTTCTC TGGAAACGGT GTCGTCATGG
101 CGACGGGAGC CTTTGCCCGT GGTGGCGCCG GTACGGTAGG CAATACACTC ACGGCAGTCG CCCAAAGCCG ATGTCGTGGC TTCAAACGGA GTGGATCTGA
201 AGGCCTCATT AGGGTCTTCG CCAGGGTTTA CCGGGCGGTT GGCATTTTCG TCAGCAGAGG CTGTTCCCGC AAAGGAGAAG CCCAGGGTCA GCAAGATGGA
301 AAGGACCAAG AGTCTTTTCA TATAATCACC TTCTCCTTAC AGGTAAAGTT CCGCAGTTGC GGAGCAATGA TATCCAAGAG GTTATTAAAT GTGCTGGCTA
401 CCTGCATAGC TACAGATGGC CCCTCTTTGC TGTCCACCTT GTTGATTTCA CCATTTGGTT TATAGGATAC CACAATTGTG CCCAAACCGG GACGGGTAAC
501 TACAGATGGC TTCCCATAGC GCTCCTCGTA CTCGATTTTA ACCACTTTCT TGGCCTGATC TGTAATGGTC GCAATACGGC CGCGGTTATC ATATGTCATA
601 TTGATCTTCT GGCCATCGCT GTTCTGGGCG AAAGAAAGAT TCCCTTTGCC ATCGTATTTG AACTGAGCGG CCTTGGTGGC TACTTTGGCA CCCTTCTCGT
701 TAAAGTAGAT GCTGTTCACC GAACTGACCT TTTTGACCTT CGGATCGTAT TCAAAGGACA TCTTCATGTT CGGTGCGGCT TTCACCTTCA CCAGGCCATC
801 AGGATAGTAT TCGTAGGAAA TACGGTCGGC GTTACGACGG ATGGAAACCG GCTTGCCGAA GACCTCATGG TAAGTGATGT CGGTGACGTT GCCGTTCACA
901 GTCGTCATCA CACGCTGCAG ATAGTATTGA CCGTCTGCGC GCTGTTGATG CCAGAATTC
EcoRI

Figure 3.

BB3 and BB4, and subjecting them to the same analysis used for BB5. Like BB5, BB3 and BB4 were of the large colony "class" of spontaneous H-I mutants, and formed plaques that were smaller and more turbid than wild-type plaques (see Chapter 1). As seen with BB5 (described above; Figure 1), recombination of pTC12 or pTC50 into the genomes of BB3 and BB4 conferred an enhanced-plaque phenotype (data not shown). These results were consistent with BB3 and BB4 having suffered mutations at the *hit* locus. To confirm that this was the case, the *Eco*RI/*Xba*I fragment containing the *hit* locus was cloned from both BB3 and BB4 and was subjected to DNA sequence analysis. The data showed that this region of the genome in both BB3 and BB4 did indeed contain a mutation (Figure 3): BB3 contained a single bp deletion 108 bp from the *Xba*I site (indicated by the closed triangle) and BB4 had a 42 bp deletion that overlapped the site of mutation in BB3 (indicated by the bracket). It is likely that the deletion in BB4 resulted from recombination between the two identical 10 bp direct repeats present in the wild-type genome beginning at positions 64 and 106 (indicated by underlining in Figure 3).

The *hit* mutations affect mutiple open reading frames. A computer search indicated that the wild-type 959 bp *Eco*RI-*Xba*I fragment contained four substantial open reading frames (ORFs) that potentially encode polypeptides (Figure 4). One of the ORFs, designated ORF2, was complete and could potentially encode a polypeptide of 11 kDa. ORFs 1, 3 and 4 were partial, and could potentially encode polypeptides of at least 23 kDa, 7 kDa and 17 kDa, respectively. ORFs 1 and 2 overlapped by 1 bp leaving no room for an intergenic promoter, suggesting that they may be part of the same operon. The mutations in BB3, BB4 and BB5 were located in a 135 bp region that affected ORFs 2, 3 and 4. Comparison of the polypeptides encoded by ORFs 1-4 to the GenBank (release 68.0, June 1991), EMBL (release 27.0, May 1991) and

Figure 4. Potential coding regions within the 959 bp *Eco*RI-*Xba*I fragment
Deletion mutations in BB3, BB4 and BB5 are indicated by the closed triangle, bracket and closed circle, respectively. All three mutations affected ORFs 2, 3 and 4.

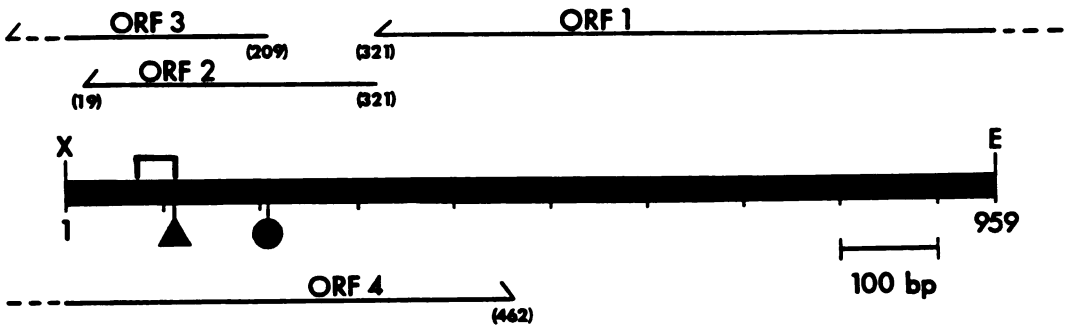

Figure 4.

SWISS-PROT (release 18.0, May 1991) data bases did not reveal any obvious similarities to previously described polypeptides. Likewise, comparison of the 959 bp *Eco*RI/*Xba*I fragment nucleic acid sequence to the same GenBank and EMBL data bases did not reveal any obvious similarities to previously reported nucleic acid sequences.

Recombination of the wild-type *hit* locus into the H-I mutants does not restore a wild-type phenotype. Recombination of the wild-type *hit* locus (carried on pTC7, pTC12 or pTC50) into BB5 and the other H-I mutants resulted in an enhanced plaque phenotype. However, as noted earlier for BB5(pTC7) and BB5(pTC12) (Chapter 1), the "enhanced plaques" appeared to be slightly smaller than those formed by the wild-type strains 109J or 109J.2(pTC3). These data suggested that the H-I recombinants were not completely restored to a wild-type phenotype. Indeed, the axenic growth characteristics of the enhanced-plaque H-I recombinants were dramatically different from the wild-type strains (Table 2). Plating 109J or 109J.2(pTC3) on PYE medium gave colonies at a frequency of 10^{-6} to 10^{-7} (compared to PFUs) that varied widely in their size and capacity to grow upon replating. Some colonies, such as those that gave rise to BB3, BB4 and BB5, were relatively large and could be picked and restreaked without difficulty. Others were small and did not give growth upon restreaking unless they were pooled with other colonies to give a large inoculum. In contrast, BB5(pTC7), BB5(pTC12), and BB5(pTC50) enhanced plaque recombinants gave colonies at a frequency of about 10^{-4} (compared to PFUs) that were relatively large, uniform in size and grew well upon replating; the colonies were indistinguishable from those formed by BB5. Thus, the axenic growth characteristics of the BB5(pTC7), BB5(pTC12), and BB5(pTC50) recombinants not like those of the wild-type, but neither were they like BB5: the plating efficiency of BB5 was essentially 1 while the plating efficiencies of the H-I recombinants were about 10^{-4}. The plating phenotypes displayed by the enhanced-plaque recombinants created by recombining pTC7, pTC12

Table 2. Plating characteristics of *hit* recombinants[a].

	109J.2(pTC3)	BB5(pTC7), BB5(pTC12) or BB5(pTC50)	BB5(pTC3)
PFUs/ml	10^9	10^9	10^9
Phenotype	large, clear	near wild-type[b]	small, turbid
CFUs/ml	10^2 - 10^3	10^5	10^9
Phenotype	variable[b]	large	large

[a] Overnight cultures of *Bdellovibrio* grown on E. coli were either plated on DNB plates containing lawns of host cells (PFUs) or on PYE plates (CFUs).

[b] See text.

and pTC50 into BB3 and BB4 were essentially the same as those described for BB5(pTC7), BB5(pTC12), and BB5(pTC50).

Recombination of mutant *hit* loci into wild-type *B. bacteriovorus* 109J yields recombinants displaying "density-dependent" axenic growth. Recombination of the mutant *hit* locus from BB5 (carried on pTC32 or pTC56) into the wild-type strain 109J.2 resulted in recombinants that formed clear plaques that were close, if not identical, in size to the plaques formed by 109J.2 (Figure 5). No recombinants were identified having the small turbid plaque phenotype of BB5. In addition, no recombinants were identified that had the "robust" axenic growth phenotype of BB5. However, the axenic growth characteristics of the recombinants were different from those of the wild-type strains: they displayed "density-dependent" growth. Overnight intraperiplasmic cultures of 109J.2(pTC32) and 109J.2(pTC56), which typically had PFU titers of about 10^9/ml, produced 10^3-10^4 uniformly sized colonies when plated on PYE at a dilultion of 10^{-1} but gave no colonies when plated at a dilution of 10^{-2}. The colonies that formed on the low dilution plate were much smaller that those formed by BB5 and developed only in close proximity to other colonies. This axenic phenotype differed from that of wild-type in that the latter gave rise to approximately 100-fold fewer colonies that were of two "classes", of which the large colony "class" could clearly grow in isolated locations. The density-dependent axenic growth of the 109J.2(pTC32) and 109J.2(pTC56) recombinants was not due to a soluble factor present in the spent growth medium of the overnight cultures as the same results were obtained with cultures that had been washed twice in fresh DNB medium. Essentially the same results were obtained when the mutant locus from BB3 (pTC52) or BB4 (pTC54) was recombined into 109J.2.

Figure 5. Mutant *hit* locus does not confer the mutant plaque phenotype upon 109J.2. Plaques formed by (A) 109J.2(pTC3) (wild-type), (B) BB5(pTC3) (H-I) and (C) 109J.2(pTC56) are compared. 109J.2 recombinants containing the *hit* locus from BB5 (pTC56) do not exhibit the BB5 plaque phenotype. pTC3 does not affect the plaque phenotype of H-D or H-I strains and is used here as a control to confer Km^r.

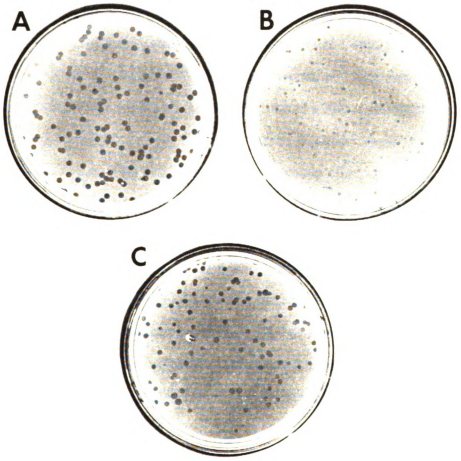

Figure 5.

DISCUSSION

Wild-type *Bdellovibrio* are obligately dependent on host cells for growth. Determining the molecular basis for this host-dependent phenotype is fundamental to an understanding of the *Bdellovibrio*-host cell interaction and, more generally, may provide additional insight into the nature of obligate parasitism. Towards these ends, we have initiated a genetic characterization of spontaneous host-independent mutants of *Bdellovibrio*. The results indicate that three independent H-I mutants suffered a mutation within a 135 bp region of the *Bdellovibrio* genome. The repeated occurence of mutations at this locus strongly suggested that it had a role in determining the H-I phenotype of the mutants. Indeed, recombination of plasmids containing the wild-type locus into the genomes of the H-I mutants had a dramatic effect on plaque phenotype (Figure 1): whereas the H-I mutants formed small turbid plaques on host cells, the H-I recombinants formed plaques that were very similar to those formed by wild-type *Bdellovibrio*. Further, recombination of mutated loci into wild-type *Bdellovibrio* had an effect on the plating phenotype of the cells: the recombinants displayed "density-dependent" axenic growth. Taken together, these data indicate that the 135 bp region altered in the H-I mutants has a significant effect on the interaction that bdellovibrios have with host cells. The locus, therefore, was desginated *hit* (host-interaction).

While it is clear that the *hit* locus is involved in determining the basic growth characteristics of *Bdellovibrio*, it is uncertain whether mutations at this locus account fully for the host-independent phenotype. Recombination of wild-type *hit* sequences into the genomes of the H-I mutants enhanced the plaque forming ability of the mutants, but the recombinants were not restored to a wild-type phenotype. Likewise, recombination of mutated *hit* loci into the genome of wild-type *Bdellovibrio* did not result in recombinants having an H-I phenotype. In both cases, the recombinants

produced had phenotypes that were "intermediate" between the wild-type and H-I strains, and distinct from one another. One potential explanation for these results is that the H-I phenotype might require mutations at both *hit* and a second locus. In this case, recombining a mutated *hit* locus into a wild-type *Bdellovibrio* or a wild-type *hit* locus into an H-I mutant would not create a wild-type or mutant genotype and the recombinants from the reciprocal crosses would be genetically distinct. Support for this scenario comes from considering the ORFs encoded by the *hit* locus.

DNA sequence analysis indicates that the *hit* mutations in each of these mutants affects three potential protein coding regions, ORFs 2, 3 and 4 (Figure 3). Presumably, one or more of these ORFs is involved in the H-I phenotype. Indeed, a role for ORF2 is suggested by the *hit* recombination experiments. Specifically, recombination of the wild-type *hit* sequences on pTC50 into the mutant *hit* locus would be expected to give two classes of recombinants, designated A and B (Figure 6). Given the site of the the *hit* mutations, most of the recombinants would presumably be of class B. Examination of the predicted ORFs in this class of recombinants indicates that only ORF2 is completely restored; ORFs 3 and 4 either contain the original spontaneous H-I mutation in on copy or are deleted at the 3' (ORF3) or 5' (ORF4) end in the other. Thus, it would appear that restoration of ORF2 is responsible for the enhanced-plaque phenotype observed in the BB3(pTC50), BB4(pTC50), and BB5(pTC50) recombinants. The involvement of ORF2 in the H-I phenotype is further supported by results obtained from the reciprocal experiment, where mutant derived *hit* sequences (pTC56) were recombined into 109J.2 In this experiment, most of the recombinants would have been of class A (Figure 6), where one copy of ORFs 3 and 4 remain unaltered. These recombinants would also be without a functional ORF2, because one copy contains the original spontaneous mutation while the other is separated from its promoter,

Figure 6. 2 classes of BB5(pTC50) recombinants. Products of single recombination events between pTC50 and the BB5 genome at sites to the left (A) and to the right (B) of the mutation in BB5 are diagrammed. Symbols: X, sites of recombination; closed circles, BB5 mutation; thick line, 959 bp EcoRI-XbaI fragment; thin line, pVKα-1; dashed line, BB5 genome; zig-zag line, ORF interruption. Restriction sites: E, *Eco*RI; X, *Xba*I.

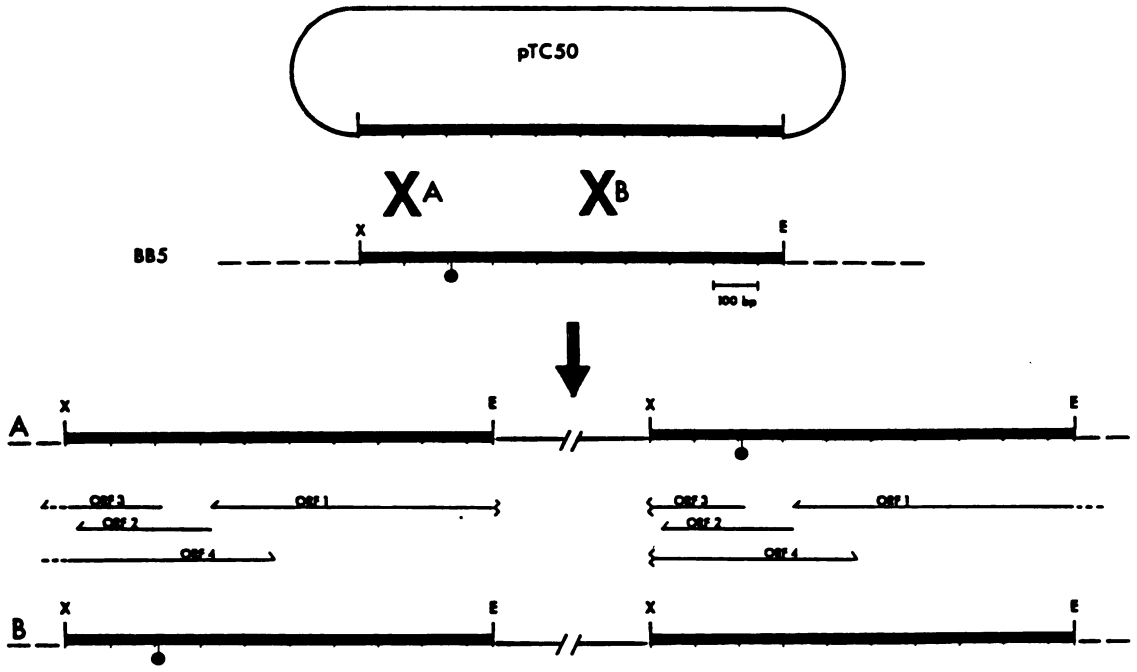

Figure 6.

assuming ORF2 is part of an operon with ORF1. Here, a defective ORF2 resulted in the density-dependent axenic growth phenotype.

Although ORF2 clearly appears to be involved in H-I growth, it does not seem to be the whole story. As just described, restoration or inactivation of ORF2 did not create wild-type or H-I recombinants, respectively. One possible explanation for these results is that, in addition to ORF2, ORF3 and/or ORF4 are also involved in the H-I phenotype. If true, then recombination of a much larger fragment of *hit* containing wild-type DNA into an H-I mutant should restore all three ORFs, assuming that all of the necessary regulatory information is contained within that larger fragment. As described in Results, pTC7, whose insert is larger than the insert in pTC50 by 5.3 kb on one side and 13.2 kb on the other, conferred the same enhanced-plaque phenotype as did pTC50 upon BB5. This argues against the involvement of ORFs 3 and 4 in the H-I phenotype. Taken in total, these results do not allow one to conclude that the described *hit* mutations are the only lesions in BB3, BB4 and BB5. Rather, as outlined above, the existence of an additional non-*hit* mutation in these three mutants may better explain the data. Of course it remains possible that *hit* mutations are solely responsible for the H-I phenotype, and that the intermediate phenotypes displayed by the recombinants are due to confounding affects that result from the creation of *hit* merodiploids, especially if the mutant *hit* alleles are not completely inactive. The development of gene replacement strategies to test *hit* alleles in a haploid state should be able to resolve whether or not BB3, BB4 and BB5 contain additional mutations outside of the *hit* locus.

The notion that the H-I phenotype of BB3, BB4 and BB5 results from two mutations offers a possible explanation for the observation of two major "classes" of H-I mutants and the density-dependent phenotype of the 109J.2(pTC56) recombinants. As described in Results, plating wild-type *Bdellovibrio* on PYE plates resulted in the formation of two general types of colonies. Some, such as those that gave rise to BB3,

BB4 and BB5, were relatively large and could be picked and restreaked without difficulty. In contrast, others were small and did not give growth upon restreaking unless they were pooled with other colonies to give a large inoculum; these did not give rise to well separated colonies. Indeed, the small colonies, at least superficially, displayed a phenotype that resembled the density-dependent phenotype of the recombinants obtained by recombining the mutated *hit* locus of BB5, carried on pTC56, into 109J.2. The possibility thus raised is that mutations at the *hit* locus might result in a partial defect in the regulation of the search phase-growth phase transition and allow for limited growth, under specialized conditions, in the absence of hosts. Mutations at the hypothetical second site(s) would then further compromise the regulation of the search-growth transition and result in robust H-I mutants.

Determining the molecular basis for the host-dependent growth phenotype of wild-type *Bdellovibrio* and the regulation of the switch between search and growth phase are essential to an overall understanding of the *Bdellovibrio*-host cell interaction. Toward this end, we have initiated a genetic analysis of spontaneous host-independent mutants and identified a locus, *hit*, that appears to be a hotspot for H-I mutations. Although the nature of the *hit* locus involvement in H-I growth is unclear, it is certain that it does play a central role in the phenotypes of BB3, BB4 and BB5. Future efforts will focus on a more detailed analysis of the *hit* locus, including a determination of the gene product(s) affected by the *hit* mutations, and the role that this gene product(s) has in *Bdellovibrio* growth and development.

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SUMMARY

The major conclusions of this Dissertation are summarized here:

1) IncQ and IncP type plasmids can be conjugally transferred from *E. coli* into *B. bacteriovorus* 109J. IncQ derivatives are maintained via autonomous replication. IncP derivatives can only be maintained in *B. bacteriovorus* 109J after integration through homologous recombination between the recipient genome and cloned *Bdellovibrio* sequences.

2) A 959 bp *Eco*RI-*Xba*I fragment of wild-type *B. bacteriovorus* 109J DNA has been identified that significantly improves the plaquing ability of three H-I mutants, BB3, BB4 and BB5.

3) H-I mutants BB3, BB4 and BB5 contain deletion mutations within the 959 bp *Eco*RI-*Xba*I fragment. Because of the ocurrence of mutations within this region, and their affect on plaque formation, this locus was named *hit*, for host-interaction.

4) Each of the *hit* mutations affects three potential coding regions (ORFs): ORF2, ORF3 and ORF4.

5) Merodiploid recombinants that contained both wild-type and mutant derived *hit* sequences displayed "intermediate" phenotypes. BB5 recombinants that contained wild-type *hit* sequences displayed the enhanced-plaque phenotype, but

still formed colonies that were indistinguishable from BB5 at reduced frequency. The reciprocal experiment, where mutant *hit* sequences were recombined into the wild-type, yielded recombinants that formed plaques that were very similar to wild-type, but also displayed a capacity for "density-dependent" axenic growth.

APPENDICES

APPENDIX A

Changes in gene expression during intraperiplasmic growth of *B. bacteriovorus* 109J.

QUESTION. Previous studies have shown that the appearance of degradative activities directed against the host cell wall and host DNA are temporally regulated during the bdellovibrio growth cycle. In this study, I have tried to expand on these observations by examining how overall patterns of bdellovibrio gene expression change, at the level of protein synthesis, throughout the entire intraperiplasmic growth cycle.

METHODS. Bdellovibrio proteins were pulse labelled with [^{35}S]methionine (1000 Ci/mmole, New England Nuclear) at various time points during synchronous growth using *E. coli* ML35 as host. The radioactive label was channeled exclusively into bdellovibrio proteins by combining a streptomycin resistant mutant of *B. bacteriovorus* 109J (109J.1) with the streptomycin sensitive ML35 in the presence of 50 μg/ml streptomycin. Fresh overnight cultures of both cell types were washed 2X and concentrated 5-fold in DNB medium, and mixed together at an MOI of 0.5 (determined by microscopy). Such two-member cultures (3 ml total volume) were incubated with shaking at 30^{o}C. Time points were pulse labelled by combining 0.1 ml culture with 1 μl [^{35}S]methionine, and incubating for 1.5 min at 30^{o}C. After labelling, cells were quickly pelleted in microfuge tubes, resuspended in 30 μl 1X sample buffer (10% glycerol, 2% SDS, 0.05% bromphenol blue, 0.2 M ß-mercaptoethanol and 64 mM Tris pH=6.8), and boiled 4 min. Labelling controls were performed such that each cell

Figure 1. SDS-PAGE analysis of in-vivo ^{35}S-labelled *Bdellovibrio* proteins. *B. bacteriovorus* 109J.1 proteins from a synchronous culture grown on ML35 in the presence of 50 μg/ml streptomycin were pulse labelled with ^{35}S-methionine and subjected to SDS-PAGE. Lane 1 shows that little host protein was labelled with ^{35}S under these conditions. Lane 2 shows the profile of bdellovibrio proteins labelled with ^{35}S in the absence of host cells. Lanes 3-12 show the profile of bdellovibrio proteins labelled with ^{35}S at various times (indicated above lanes) during synchronous culture. (A), (B), and (C) indicate examples of bdellovibrio proteins labelled during the early, late and middle stages of intraperiplasmic growth.

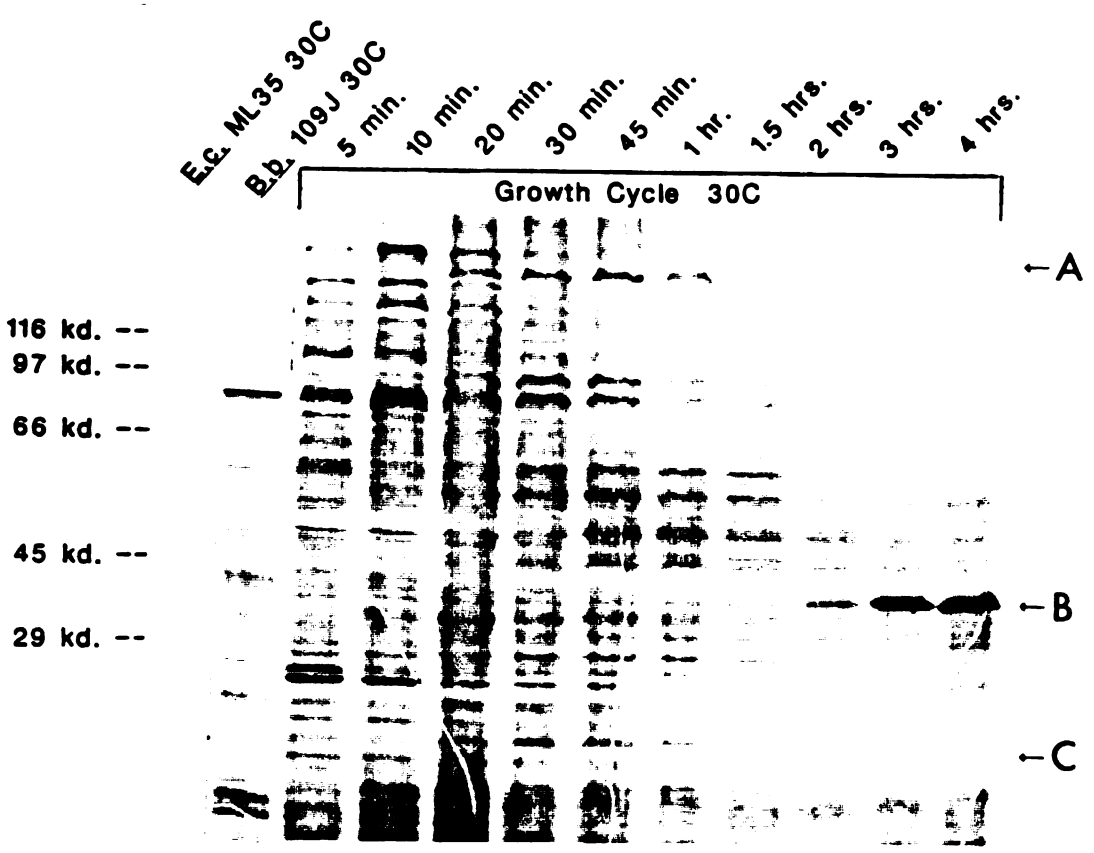
E.c. ML35 30C
B.b. 109J 30C
5 min.
10 min.
20 min.
30 min.
45 min.
1 hr.
1.5 hrs.
2 hrs.
3 hrs.
4 hrs.
Growth Cycle 30C
116 kd. --
97 kd. --
66 kd. --
45 kd. --
29 kd. --
←A
←B
←C

Figure 1.

type was present in numbers equivalent to that of mixed culture. Samples were electrophoresed in gradient acrylamide gels (8-14.5%).

RESULTS AND DISCUSSION. Figure 1 shows changes in overall patterns of protein synthesis during the 3-4 hr intraperiplasmic growth cycle. Inspection of lane 1 shows that, in the presence of streptomycin, very little label was incorporated into host cell proteins, indicating that the vast majority of labelled protein seen in Figure 1 was of bdellovibrio origin. A striking feature of this analysis is the change in bdellovibrio gene expression that occurred within 5 min of mixing of the two cell types (compare lanes 2 and 3). Lane 3 shows the induction of at least 6 different proteins, even though the attacking bdellovibrios had not yet penetrated the host cells. This observation indicates that information concerning the presence of the host is quickly transmitted to effectors of gene expression.

Further analysis of Figure 1 shows that a number of bdellovibrio proteins are produced at higher levels during specific periods of growth. Examples of enhanced production during the early, middle and late stages of growth are represented by the bands labelled A, C and B, respectively. Production of A is seen at the earliest time point (5 min), and A essentially disappears by 60 min. Levels of C are highest from 20 min through 2 hrs. Production of B begins to increase after 2 hrs, and continues to elevate until the end of growth.

The changes in bdellovibrio gene expression seen in Figure 1 are reproducible, having been observed in three separate experiments (data not shown). Thus, the changes seen here probably reflect a developmental program of gene expression expressed during growth in the intraperiplasmic environment.

APPENDIX B

Other methods of introducing DNA into *Bdellovibrio*

Prior to the development of a conjugation system for *Bdellovibrio* (described in Chapter 1), other methods of plasmid transfer were tested. I attempted to transform *B. bacteriovorus* 109J with the IncQ derivative pSUP204 (4) and the IncP derivative pRK290 (2) using $CaCl_2$ (1) and RbCl (3) based transformation procedures. Each method was tested twice, and on no occasion did either of these procedures yield tetracycline resistant (5 - 25 μg/ml) transformants (using pRK290 or pSUP204) or chloramphenicol resistant (5 - 25 μg/ml) transformants (using pSUP204). At the time these experiments were conducted, I did not know that these plasmids do not express tetracycline resistance in *B. bacteriovorus* 109J (see Chapter 1). However, the fact that transformation of pSUP204 did not yield any chloramphicol resistant transformants indicated that neither method was effective because 1) these procedures did not render *B. bacteriovorus* 109J competent for DNA uptake, or 2) because the plasmid DNA was propagated in *E. coli*, it was immediately degraded in *Bdellovibrio* due to inappropriate methylation patterns.

In addition to the attempts at transformation, I also tried to introduce pSUP204 and pRK290 into *B. bacteriovorus* 109J by electroporation. A field strength of 10 kV/cm failed to yield any transformants using the range of antibiotic concentrations described above. Additional information concerning the electrical conditions employed in these experiments was not available, since I was using a "homemade" device constructed in Chris Somerville's laboratory. As was the case with the transformation experiments

described above, these experiments were conducted without the knowledge that tetracycline resistance is apparently not an effective selection for the maintenance of plasmids in *B. bacteriovorus* 109J. The inability to obtain chloramphenicol resistant transformants after introduction of pSUP204 by electroporation was possibly due to 1) inappropriate electroporation conditions or 2) degradation of plasmid DNA that contained inappropriate, *E. coli* specific methylation patterns.

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APPENDIX C

Delivery of Tn*5* into *Bdellovibrio bacteriovorus*

The development of a conjugation system for *Bdellovibrio* (described in Chapter 1) created a potential means of delivering transposons into the *Bdellovibrio* genome. In mating experiments that employed the suicide delivery vehicle pSUP2021 (3) and *E. coli* SM10 (2) as donor, Tn*5* was delivered into H-I mutants of *B. bacteriovorus* 109J at a frequency of approximately 10^{-8}. Transposition from pSUP2021 into the H-I mutant genome was confirmed by Southern analysis (data not shown). In contrast to these results, I was unable to demonstrate transposition of Tn*5* from pSUP2021 into the wild-type *B. bacteriovorus* 109J genome. It is known that Tn*5* transposition is inhibited by Dam methylation (4), and so I tested both wild-type and H-I mutant DNA for Dam methylation by restriction digestion analysis with the Dam-sensitive *Mbo*I and the Dam-insensitive *Sau*3AI. These enzymes digested the two DNAs equally well which indicated that Dam methylation was not inhibiting Tn*5* transposition in these experiments. Therefore, I cannot explain why I was unable to demonstrate Tn*5* transposition from pSUP2021 into the wild-type *B. bacteriovorus* genome. In addition to the difficulties encountered in delivering Tn*5* by conjugation, I was unable to obtain kanamycin resistant, Tn*5* containing bdellovibrios by way of P1::Tn5 infection, using conditions that had been successful for the delivery of Tn*5* into *Myxococcus xanthus* (1). Because of the low frequency of transposition in H-I mutants and the complete lack of transposition in the wild-type, I did not utilize transposon mutagenesis as a tool in the genetic analysis of *B. bacteriovorus* 109J. These results, however, should not be

taken to mean that other Tn*5* delivery vehicles, or the use of other transposons would be unsuccessful.

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APENDIX D

Trans complementation experiments

In addition to the recombinational experiments involving the *hit* locus, I also examined the affects of wild-type and mutated *hit* loci *in-trans*. The wild-type 5.6 kb *Bam*HI *hit* containing fragment (described in Chapter 1), and the equivalent region from BB3, BB4 and BB5 (described in Chapter 2) were cloned into the *Bam*HI site of the IncQ derivative pMMB33 (1) and shown to be stably maintained after repeated sub-culture in *E. coli*. Each fragment was cloned in both orientations to create the following constructs: pTC8 and pTC9 (wild-type 5.6 kb BamHI fragment); pTC42 and pTC43 (5.6 kb BamHI fragment from BB3); pTC44 and pTC45 (5.6 kb BamHI fragment from BB4); and pTC46 and pTC47 (5.6 kb BamHI fragment from BB5). The 5.6 kb inserts in pTC9, pTC42, pTC44 and pTC46 were oriented such that the promoter of the vector-borne streptomycin resistance gene would promote transcription across ORF2 (assuming the promoter was active in *Bdellovibrio*). The 5.6 kb inserts in pTC8, pTC43, pTC45 and pTC47 were cloned in the opposite orientation. In general, maintenance of pMMB33, as well as other constructs derived from it, resulted in plaques and colonies that were only about 50% the size of those formed by the recipients alone, which made it more difficult to assess the phenotypic affects of these constructs.

The constructs containing mutated *hit* sequences were tested only once for affects on 109J.2, BB3, BB4 and BB5, yielding the following results: 1) all the constructs (pTC42-pTC47) conferred the density-dependent axenic growth capability (described in

Chapter 2) upon 109J.2, and yet had no apparent affect on the plaque phenotype and 2) none of these constructs had any affect on plaque or colony formation by BB3, BB4 or BB5. One potential explanation for these results is that the mutated *hit* loci are dominant over the wild-type locus, at least when present on a high copy number plasmid. It also suggests that the mutated *hit* loci can be expressed from promoter activity present within the 5.6 kb *Bam*HI fragment. Alternatively, the BamHI fragment might contain a promoter region that titrates out regulatory factors, and thus effectively creates a *hit*$^-$ cell (turns off the wild-type copy of *hit* in the chromosome). Finally, the data suggest that a mutation at *hit*, by itself, is not sufficient for the full H-I phenotype displayed by BB3, BB4 and BB5.

The transfer of pTC8 and pTC9 (wild-type *Bam*HI fragment) into the H-I mutants was done twice, and the following affects were observed: 1) plaque formation by BB3 was not enhanced by either plasmid, 2) plaque formation by BB4 was slightly enhanced by both plasmids 3) plaque formation by BB5 was moderately enhanced by pTC9 but not pTC8. The plaque-enhancement observed in BB4 and BB5 was much less than that conferred by recombining pTC12 into the same strains. These results suggest that the mutated *hit* alleles have a co-dominant relationship with the wild-type allele.

Taken together, these results suggest that the mutant alleles of *hit* can produce products that interact with the wild-type *hit* gene product(s), and prevent its activity. Further analysis will be required to definitively show whether this is or is not the case.

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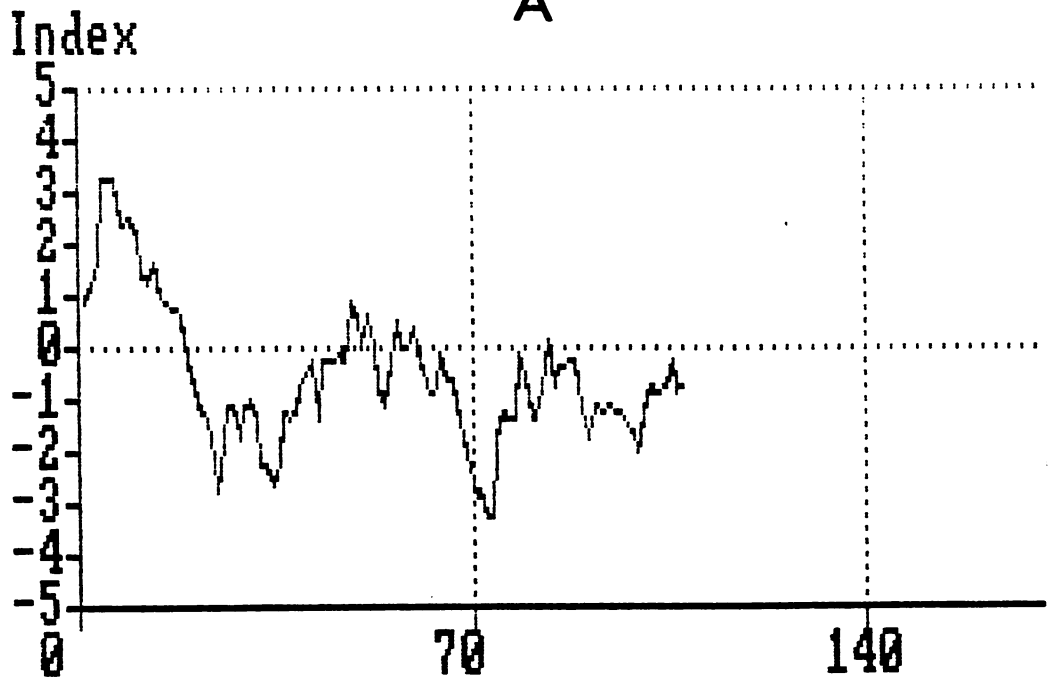
APPENDIX E

Characterization of the putative ORF2 gene product

Because the genetic experiments described in Chapter 2 indicated that ORF2 of the *hit* locus is involved in the plaque-enhancement of H-I mutants BB3, BB4 and BB5, I have conducted further computer analysis of the putative 101 amino acid, 10.59 kDa ORF2 gene product, using PROSIS software (Hitachi). The results of a Kyte and Doolittle hydrophobicity analysis (2) are shown in Figure 1A. The data indicate that the majority of the putative gene product is hydrophilic. However, the N-terminal 18 amino acids are quite hydrophobic raising the possibility that ORF2 contains an N-terminal signal peptide for transport across the cytoplasmic membrane. According to the consensus signal peptide cleavage site, as defined by Oliver (3), the serine at position 16, and the alanines at positions 18, 21 and 23 (Figure 1B), are possible sites for the C-terminal residue of the signal peptide. Also in agreement with consensus leader peptide characteristics (3), the 18 N-terminal amino acid residues of ORF2 assume an α-helical or ß-sheet secondary structure (Figure 1B) according to the rules developed by Chou and Fasman (1). These characteristics of the putative ORF2 polypeptide strongly suggests that ORF2 is a "real" *Bdellovibrio* gene. If proven to be so, it is likely that the ORF2 gene product is periplasmically located or secreted, since the mature polypeptide is of a hydrophilic nature.

A



B

```
          10        20        30        40        50        60        70
      MKRLLVLSILLTLGFSFAGTASADENANRPVNPGEDPNEAFRSTPFEATTSALGDCRECIAYRTGATTGK

HELIX hhhhhhhhhhhhhhhhhH
SHEET SsSSSSSSSSSSSSSSs                                SSSSSSSSS SSSSSSSsSSSS
TURN   T                        T   T   TTTTTTT   TT                    T     T
COIL                       cccccc ccc ccc       ccc            c             c


          80        90       100       110
      GSRRHDDTVSREIKGSSATPGGSEKAGTGRQ*SHLSR

HELIX
SHEET
TURN  TTT    T   T   TT     TTTT     TT
COIL     ccc ccc ccc  cccc    ccccc   ccccc
```

Figure 1. Computer analysis of ORF2

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