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Symbiosis-Independent and Symbiosis-Incompetent Mutants of *Bdellovibrio bacteriovorus* 109J

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Symbiosis-independent (S^{in}) mutants were isolated from the symbiosis-dependent and symbiosis-competent ($S^{d}comp^{+}$) *Bdellovibrio bacteriovorus* 109J. Independently isolated S^{in} mutants were examined for their symbiosis competence and most were found to be *comp*$^{+}$. Bdellovibrios *comp*$^{-}$ were selected from the $S^{in}comp^{+}$ mutants. The $S^{in}comp^{+}$ bdellovibrios are always at a selective disadvantage, either against $S^{in}comp^{-}$ bdellovibrios (in organic medium) or against $S^{d}comp^{+}$ bdellovibrios (in buffer with *Escherichia coli* cells).

Bdellovibrio was originally described as a "predatory, ectoparasitic and bacteriolytic" bacterium (13). An ambiguity in the definition of bdellovibrios has persisted until the present time. Starr (12) recently suggested that the relationship between bdellovibrios and the other bacteria required for their growth could best be regarded as an example of symbiosis by deBarry's definition.

The dependence on another bacterium for normal growth and the competence to establish and maintain a specific association with it are two unique properties of *Bdellovibrio*. Thus, the wild-type bdellovibrios can be described as symbiosis dependent (S-D) and symbiosis competent (S-C) as suggested by Starr (12), or $S^{d}comp^{+}$.

Symbiosis-independent (S^{in}) mutants of *Bdellovibrio* were isolated as early as 1963. They were then named "saprophytic," later "host-independent" and a variety of other designations (12). The disadvantage of the different designations was that they did not clearly distinguish between the two properties of *Bdellovibrio*. Thus, a "host-independent bdellovibrio" was assumed to be incapable of penetrating and growing inside other bacteria, though the capacity to grow in bacteria-free medium does not necessarily imply the loss of the capacity to grow in the periplasmic space of other bacteria. *Bdellovibrio* mutants isolated up to now have differed from their parent in their symbiotic dependence or in their symbiotic competence or both. We suggest the following nomenclature for the three classes of *Bdellovibrio* mutants which have been described: (i) $S^{in}comp^{+}$, mutants that have been described as being facultative (2, 11) and that can establish a symbiotic relationship with other bacteria but do not depend on them for normal growth; (ii) $S^{in}comp^{-}$, mutants that have been described as being nonparasitic (14) or nonsymbiotic (12) and that have lost their symbiotic competence but gained the capacity for independent growth; (iii) $S^{d}comp^{-}$, mutants that are conditional and that are competent at the permissive temperature but incompetent at higher, restrictive temperatures (3, 10).

In the present study we isolated mutants of types (i) and (ii) from a single wild-type *Bdellovibrio* strain (109J) and investigated the relationship between them.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Bdellovibrio* 109J (streptomycin resistant) and *Escherichia coli* B have been described previously (16). PY medium contained (grams/liter): peptone, 10; and yeast extract, 3. ENB medium contained (grams/liter): nutrient broth, 8; Casamino Acids, 3; and yeast extract, 1. ENB medium was supplemented in certain cases with an extract prepared from *E. coli* cells since, according to Ishiguro (7), *E. coli* cells contain a factor required for optimal growth of S^{in} bdellovibrios. This growth-initiating extract was prepared from frozen *E. coli* cells (2.4 g) thawed in 10 ml of distilled water and treated with deoxyribonuclease (50 μg/ml) in the presence of $MgSO_4$ (10^{-3} M) for 30 min at 37 C; the suspension obtained after low-speed centrifugation was introduced into the medium (8.3 ml/liter) before autoclaving. Plates of this medium (ENB-E) as well as of ENB and PY were usually supplemented with 50 μg of streptomycin per ml. DNB medium and TM buffer were as described by Varon and Shilo (16).

Mutant selection and maintenance. Spontaneous S^{in} mutants were selected by plating 2×10^{8} to 4×10^{8}

Bdellovibrio 109J cells on ENB-E plates. Within a week of incubation at 30 C colonies appeared and ranged in number between 200 and 400. Mutants obtained from separate cultures were picked and subcultured on the same medium. The wild type and S^{in}*comp*$^+$ mutants were grown in liquid cultures with *E. coli*, centrifuged, and stored in DNB medium at 4 C for several months; inocula for fresh cultures were withdrawn directly from these suspensions.

Spontaneous S^{in}*comp*$^-$ mutants were selected from S^{in}*comp*$^+$ cultures by repeated passages on ENB slants every 3 to 5 days. S^{in}*comp*$^-$ mutants were maintained by transfer on ENB slants every fortnight.

Axenic growth of S^d bdellovibrios. Axenic growth of S^d bdellovibrios was tested with samples of 2×10^7 cells deposited on plates containing the host-free extract medium described by Horowitz et al. (6) and 1% agar. Treatment of the extract with ribonuclease, Pronase, or deoxyribonuclease was done as described previously (6).

Viable counts of S^d and S^{in} bdellovibrios. S^d*comp*$^+$ bdellovibrios were counted by plaque assay on DNB plates; S^{in}*comp*$^-$ bdellovibrios were counted as colonies on ENB-E medium or on PY medium. S^{in}*comp*$^+$ bdellovibrios were counted by both plaque assay and colony counts.

The number of S^d bdellovibrios in a mixture containing both S^d*comp*$^+$ and S^{in}*comp*$^+$ was calculated by subtracting the number of colonies from the total number of plaques. The number of *comp*$^+$ bdellovibrios in a mixture containing both S^{in}*comp*$^-$ and S^{in}*comp*$^+$ was calculated by subtracting the number of plaques from the total number of colonies.

RESULTS

Description of S^{in} mutants. Table 1 summarizes the growth characteristics of the symbiosis-independent mutants isolated from the wild-type 109J. Though incapable of growth on autoclaved bacteria, *Bdellovibrio* 109J grows in the presence of cell extract prepared from *E. coli* or other bacteria (6, 8). Treatment of the extract with ribonuclease or Pronase abolishes its activity (6). No other medium is known to support normal growth of *Bdellovibrio* 109J. All

TABLE 1. *Growth of different Bdellovibrio 109J phenotypes on various substrates*[a]

Substrate	S^d*comp*$^+$ (wild type)	S^{in}*comp*$^+$	S^{in}*comp*
Living cells	+	+	−
Autoclaved cells	−	+	+
Bacterial extract	+	+	+
Ribonuclease-treated extract	−	+	+
Pronase-treated extract	−	+	+
ENB-E medium	−	+	+

[a] Growth was examined on plates as plaques (on lawns of living cells) or as colonies (on all the other substrates).

S^{in} mutants examined differed from the wild type in their ability to grow on autoclaved bacteria, on ribonuclease- or Pronase-treated bacterial extracts, and on complex media containing commercial yeast extract in combination with peptone (PY medium) or nutrient broth and amino acids (ENB medium). On a lawn of living bacteria, S^{in}*comp*$^+$ bdellovibrios formed plaques with a central colony (2, 14), whereas S^{in}*comp*$^-$ did not form plaques.

The growth cycle of all S^{in} mutants either in ENB or PY medium or in the periplasmic space of the symbiont is, on the whole, similar to the growth of the wild type, as has been observed for *Bdellovibrio* UK12 (1). In all cases the small single-unit cell elongates and grows into a filament several cell units in length; at the end of the cycle the filament divides by multiple fission into several daughter cells.

Rapid motility, which is a typical characteristic of S^d bdellovibrios, is very rare in cultures of S^{in} mutants. Samples of S^{in} cultures growing axenically in ENB or PY medium were taken at different stages of growth and viewed in a phase microscope directly or after centrifugation and resuspension in a dilute medium (DNB) or buffer (TM). Ten mutants were examined in this way, and none resembled the wild type in motility: only occasionally could a motile cell be observed. Electron microscope observations of negatively stained preparations showed that, unlike the wild-type cells, most cells of S^{in} mutants were aflagellate. The few cells that carried flagella were always the short, single-unit cells; bdellovibrios that grew into a short or long spiral were always aflagellate. Unattached flagella appeared in every preparation and could be collected from the culture supernatant by a 60-min centrifugation at $120{,}000 \times g$. On the basis of these observations, we assume that the mutant cells possess normal, functional flagella, but for a very short period of time in their life cycle, after which the flagella are shed.

Examination of the symbiosis competence of the S^{in} mutants revealed that, after three passages on ENB-E plates (required for their purification), 92% of the mutants were *comp*$^+$, i.e., they were capable of plaque formation on lawns of living cells. After repeated transfers in axenic culture, progressively more mutants lost their plaque-forming capacity and became *comp*$^-$ (Table 2).

In a two-membered culture (with *E. coli*) incubated in buffer, in which symbiosis competence is a prerequisite for growth, selection pressure favors the *comp*$^+$ mutants. However, upon 40 passages under such conditions, two of

TABLE 2. *Selection of $S^{in}comp^-$ phenotypes upon repeated passages of $S^{in}comp^+$ mutants in ENB medium*[a]

Passage no.	No. of S^{in} mutants	No. of *comp*$^+$ mutants	*comp*$^-$ phenotypes (% of total S^{in})
3	37	34	8.0
5	39	33	15.0
10	31	22	28.7
20	29	16	44.9
30	28	12	57.2
40	28	4	85.7
50	28	4	85.7
60	28	4	85.7

[a] Passages were on ENB slants every 3 to 5 days. Samples were withdrawn from the slants and plated after dilution on ENB-E plates (colonies) and on lawns of *E. coli* (plaques).

the four $S^{in}comp^+$ mutants examined reverted to the S^d phenotype. The two revertants were, like the wild type, incapable of colony formation either on ENB-E medium or on autoclaved bacteria.

Growth kinetics of S^{in} bdellovibrios in axenic cultures. The growth rates of $S^{in}comp^+$ and $S^{in}comp^-$ mutants in ENB medium were followed by means of absorbancy measurements and viable counts. Although different *comp*$^+$ mutants differed in their growth rate, they grew more slowly than the *comp*$^-$ mutants derived from them or from other *comp*$^+$ mutants, whether in axenic cultures (Fig. 1) or in a mixed culture containing both types of mutants. As a result, during several transfers of the mixed culture, the proportion of *comp*$^+$ cells gradually decreased until the population consisted mainly of *comp*$^-$ cells. A "fitter" *comp*$^+$ mutant obtained by successive transfers competed successfully with the *comp*$^-$ mutant for at least 13 transfers (Fig. 2).

Thus, a spontaneous mutation *comp*$^+$ → *comp*$^-$, followed by a selection process such as illustrated in Fig. 2, could account for the loss of plaque-forming capacity of $S^{in}comp^+$ populations repeatedly transferred under conditions that allow asymbiotic growth of bdellovibrios (Table 2).

Growth kinetics of comp$^+$ bdellovibrios in two-membered cultures. The growth rate of the S^{in} mutants was slower than the growth rate of the S^d parent, either when grown separately on *E. coli* or in a mixed culture containing both wild-type and mutant bdellovibrios and *E. coli* (Fig. 3). This difference in the growth rate of the two kinds of bdellovibrios probably explains the disappearance of S^{in} cells from a mixed culture transferred repeatedly (Fig. 4).

Differences in growth rates between the two symbiotically growing bdellovibrios seem to be due to differences in the efficiency of symbiosis establishment rather than differences in growth rate per se. Growth itself, as measured in one-step growth experiments, was similar in rate in both strains; the difference was within the range of variation obtained in repeated experiments with a single strain. The attachment efficiency, on the other hand, is significantly reduced in the S^{in} mutants and is still further reduced after incubation in a fresh medium (Fig. 5). Phase microscopy showed that the decrease in attachment efficiency in the mutant cells is accompanied by a loss of motility and enlargement of the cells.

Infection capacity of comp$^-$ mutants. We have defined *comp*$^-$ mutants as bdellovibrios incapable of plaque formation on *E. coli* lawns (ratio of plaque-forming units/colony-forming units being less than 10^{-3}). However, when examined in suspension, all of the mutants showed a significant capacity for infection and symbiotic growth (Table 3). Though most cells were nonmotile, a significant proportion (up to 20% in 40 min in mutant D-42N) attached to *E. coli* and proceeded to penetrate into the periplasmic space and grow there.

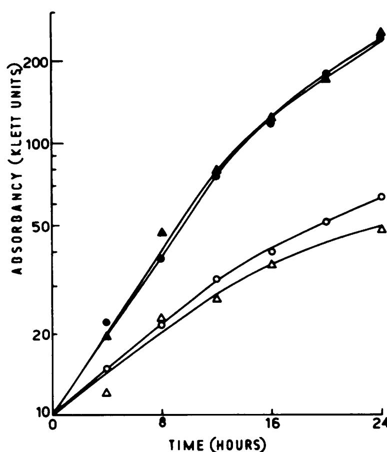

FIG. 1. *Growth of S^{in} mutants in ENB medium. Logarithmic-phase inocula were introduced into 100-ml side-arm flasks containing 20 ml of ENB medium (at an initial concentration of 10 Klett units) and incubated on a rotary shaker at 30 C. Absorbancy was measured in a Klett-Summerson photometer with filter no. 42. $S^{in}comp^+$ mutants C-4715 (○) and D-4210 (△); $S^{in}comp^-$ mutants G-47N (●) and G-42N (▲).*

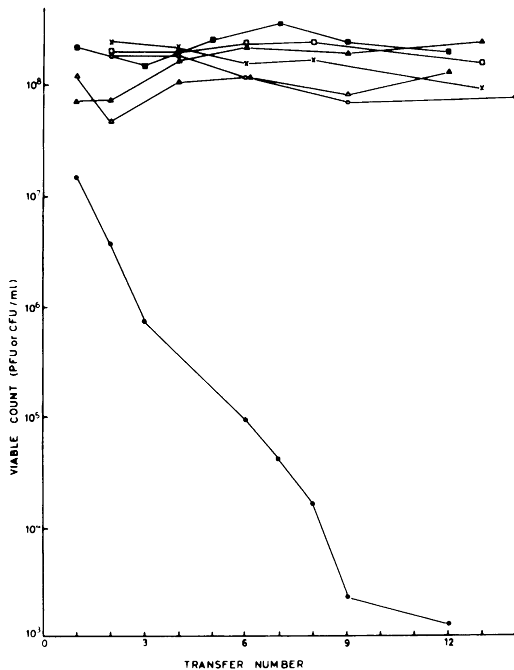

FIG. 2. *Competition between S^{in}comp$^+$ and S^{in}-comp$^-$ mutants in ENB medium. Inoculation and incubation were as described in the legend to Fig. 1. When cultures grew to concentrations of about 100 to 200 Klett units, inocula were transferred again into fresh media to give a concentration of 10 Klett units; this was repeated 12 to 14 times, and each time viable counts were made. Symbols: ●, S^{in}comp$^+$ mutant G-4820 (grown in mixed culture with S^{in}comp$^-$ mutant G-48N derived from it); △, S^{in}comp$^+$ mutant G-4820 (grown alone), ×, S^{in}comp$^-$ mutant G-48N (grown alone); ○, total number of S^{in}comp$^+$ mutant G-4820 and S^{in}comp$^-$ mutant G-48N (grown in a mixed culture); ▲, S^{in}comp$^+$ fitter mutant G-4835 (grown in a mixed culture with S^{in}comp$^-$ mutant G-48N); ■, S^{in}comp$^+$ fitter mutant G-4835 (grown alone); □, total number of S^{in}comp$^+$ fitter mutant G-4835 and S^{in}comp$^-$ mutant G-48N (grown in a mixed culture). CFU, Colony-forming units; PFU, plaque-forming units.*

DISCUSSION

S^{in} mutants express metabolic potentials lacking in the parent: they can grow on a complex commercial medium, on ribonuclease-treated bacterial extract, or on substrates released from autoclaved bacteria. Several mechanisms have been described by which different bacteria gain the ability to grow on a novel compound (4): (i) derepression of a previously inducible enzyme (mutation in a regulatory gene); (ii) change in the affinity of a preexisting constitutive enzyme toward the new compound (mutation in a structural gene); (iii) change in the specificity of the inducer; (iv) acquisition of permeability to a metabolized compound to which the parent was impermeable; and (v) decrease in sensitivity to growth-inhibiting metabolites normally produced from the new compound.

The available data do not allow us to choose among these or other possible mechanisms. A control mechanism that prevents growth of S^d bdellovibrios outside the periplasmic space of suitable bacteria has been suggested by Shilo

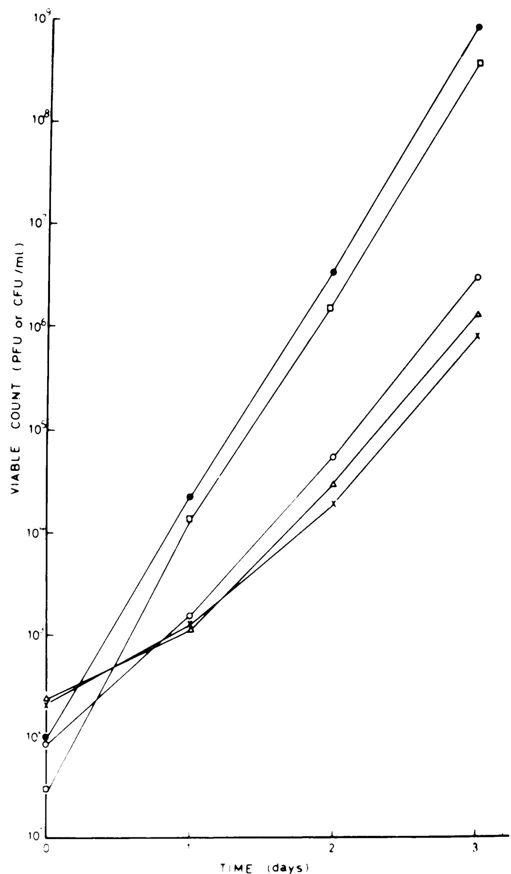

FIG. 3. *Growth rate of S^dcomp$^+$ Bdellovibrio 109J and S^{in}comp$^+$ mutant no. C-3910 in two-membered culture with E. coli (10^9 cells/ml) suspended in TM buffer. The cultures were incubated on a rotary shaker at 30 C, and samples were withdrawn for viable counts. CFU, Colony-forming units; PFU, plaque-forming units. Symbols: ●, plaques of S^dcomp$^+$ (growing with E. coli); ○, plaques of S^{in}comp$^+$ (growing with E. coli); △, colonies of S^{in}comp$^+$ (growing with E. coli); □, calculated plaques of S^dcomp$^+$ (growing in a mixed culture with S^{in}comp$^+$ and E. coli); ×, colonies of S^{in}comp$^+$ (growing in a mixed culture with S^dcomp$^+$ and E. coli).*

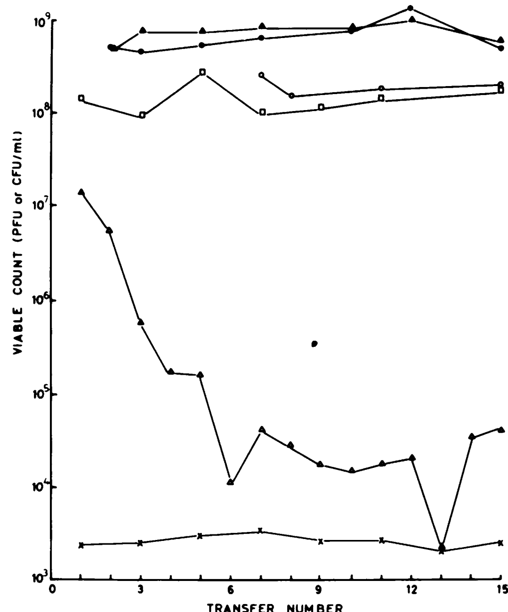

FIG. 4. *Competition between $S^{d}comp^{+}$ Bdellovibrio 109J and $S^{in}comp^{+}$ mutant no. C-3910 in a mixed culture. At first 3 × 10^{8} plaque-forming units (PFU) of each strain per ml were inoculated into a suspension of 10^{9} E. coli cells per ml of TM buffer. The E. coli cells lysed after approximately 24 h (at 30 C); 1 ml of lysate was used to infect a fresh E. coli suspension (as above). Viable counts were determined at each transfer in each flask. CFU, Colony-forming units. Symbols: ●, plaques of $S^{d}comp^{+}$ (grown with E. coli); ×, mutation count of $S^{in}comp^{+}$ cells in a population of $S^{d}comp^{+}$ (grown with E. coli); O, plaques of $S^{in}comp^{+}$ (grown with E. coli); □, colonies of $S^{in}comp^{+}$ (grown with E. coli); ▲, calculated plaques of $S^{d}comp^{+}$ (grown in a mixed culture with $S^{in}comp^{+}$ and E. coli); Δ, colonies of $S^{in}comp^{+}$ (grown in a mixed culture with $S^{d}comp^{+}$ and E. coli).*

(10). Since S^{d} bdellovibrios are incapable of deoxyribonucleic acid synthesis though they can generate energy (5) and synthesize other macromolecules (16), such a control mechanism may operate on the deoxyribonucleic acid level. A repressor that prevents deoxyribonucleic acid synthesis in the wild type unless inactivated by a component from the inhabited bacteria could be inactivated in S^{in} mutants by a variety of mechanisms, some of which are listed above. The acquisition of a new metabolic potential is often followed by a selection of fitter variants, which grow faster on the new compound (4). This phenomenon was also observed in the case of our mutants: their growth rate in ENB medium and in a two-membered culture becomes faster after repeated transfers in the same medium. This may explain why $S^{in}comp^{-}$ mutants derived from $S^{in}comp^{+}$ populations grow faster than their parents and dominate the population (Fig. 1 and 2, Table 2).

However, one can speculate on another explanation for the difference in growth rate between $comp^{+}$ and $comp^{-}$ bdellovibrios. Mutations to $comp^{-}$ probably involve loss of functions having to do with the special symbiotic growth of *Bdellovibrio*. Mutants described so far include motility mutants (9) and mutants defective in attachment or penetration (3, 10, 14) or intraperiplasmic growth (3). One would expect to find different mutants for each of the stages of the symbiotic way of life, as well as mutants in regulatory genes that coordinate the different functions. These functions are not needed when the bdellovibrios are grown on rich media. It was shown by Zamenhof and Eichhorn (17) a few years ago that, on rich media supplied with final metabolites, auxotrophs have a selective advantage over prototrophs, and the latter have a selective advantage over derepressed mutants. These results were attributed to the useless production of metabolites by the prototroph and even more by the derepressed strain. If this is a general phenomenon, one would expect $comp^{-}$ bdellovibrios to grow faster than corresponding $comp^{+}$ bdellovibrios. To examine this it is essential to use strains closely related

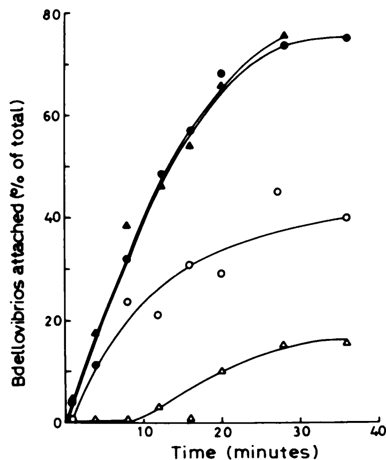

FIG. 5. *Attachment of $S^{d}comp^{+}$ Bdellovibrio 109J and $S^{in}comp^{+}$ mutant C-3910 to E. coli cells. Half of the suspension of each bdellovibrio strain was used immediately (time zero) for attachment test, whereas the other half was incubated for an additional 3 h in DNB medium before a second attachment test. Symbols: ●, wild type at time zero; O, mutant at time zero; ▲, wild type at time 3 h; Δ, mutant at time 3 h. The two bdellovibrio strains and E. coli were grown, prepared for the experiment, and tested for attachment as described previously (15).*

TABLE 3. *Interaction of Bdellovibrio 109J and certain S^{in} mutants with E. coli*[a]

Strain	Flagella at 0 min	PFU/CFU[b] at 0 min	Attachment at 20 min	Penetration at 40 min	Intraperiplasmic elongation at 4.5 h	*E. coli* lysis at 22 h	Flagella at 22 h
109J	+++	10^5	+++	+++	E[c]	+++	+++
C-3910	+	1	++	++	E	+++	+
A-9N	−	10^{-4}	++	+	+	++	−[d]
B-27N	−	10^{-4}	+	+	+	+	−
C-39N	−	10^{-4}	++	+	+	++	−
D-42N	±[e]	10^{-4}	++	+	+	++	±[e]

[a] Bdellovibrios (10^9 cells/ml) and *E. coli* (10^9 to 1.5×10^9 cells/ml) were washed, mixed together in TM buffer containing 100 μg of streptomycin/ml, and incubated in a shaking bath at 30 C. Samples were taken periodically for phase microscopy. Flagella were examined in electron micrographs of negatively stained preparations.

[b] PFU, Plaque-forming units; CFU, colony-forming units.

[c] By this time a second growth cycle had started in the 109J culture so that elongation had already occurred earlier.

[d] Numerous unattached flagella observed in the supernatants of all five S^{in} mutants.

[e] The few flagella are abnormal.

genetically. Our *comp*$^-$ strains could not be easily compared to their *comp*$^+$ parent since they may differ in more than a single mutation. As pointed out by Hegeman and Rosenberg (4), the auxotrophs should ideally be compared with prototrophic transformants or transductants.

The stability of a S^{in}*comp*$^+$ bdellovibrio population in rich media depends on the frequency of *comp*$^-$ mutations and on the selective advantage of the *comp*$^-$ mutant. Of 28 mutants examined by us, 50% were converted to *comp*$^-$ after 20 transfers. A few of the isolates were transformed into *comp*$^-$ much sooner, whereas a few others remained stable even after 60 transfers.

The existence of S^{in}*comp*$^-$ bdellovibrios in nature is difficult to determine at present because of the difficulty of isolating *Bdellovibrio* colonies from a mixed population. Such isolation demands a special selective method to compensate for the requirement for a rich medium and the slow growth of *Bdellovibrio*. Of the two known *comp*$^+$ forms, S^{in}*comp*$^+$ and S^{d}*comp*$^+$, it has been assumed that the natural form is the S^d. However, bdellovibrio isolates were not examined for symbiosis dependence immediately after isolation. We have shown that repeated transfers in two-membered cultures in the laboratory select for the S^d form; therefore, it is possible that S^d bdellovibrios predominated by the time they were examined even though S^{in} cells might have been originally isolated. On the other hand, the selective advantage of S^d bdellovibrios may also exist in natural environments.

The selective disadvantage of S^{in} bdellovibrios in two-membered cultures seems to be due to their inefficient attachment compared to that of S^d (see Fig. 5). A feature common to all S^{in} bdellovibrios derived from 109J is the ease with which they shed their flagella. The proportion of flagellated or motile cells in S^{in} populations is always low compared with that of S^d populations. The few flagellated cells are always single-unit cells, whereas the spirals of various lengths are aflagellated and nonmotile. Preliminary experiments with wild-type cells growing in host-free extract, or S^{in} mutants growing in ENB medium in the presence of low doses of penicillin, indicated that elongation is initiated polarly, apparently at the flagellar pole, and is associated with the loss of flagella. S^d cells in the two-membered cultures grow only after they penetrate the periplasmic space of their symbiont, whereas S^{in} cells can grow outside the symbiont. If growth is indeed accompanied by loss of flagella, this might explain the slower attachment rate and the fact that additional growth decreases attachment (Fig. 5). Poor motility is also common among S^{in} mutants of other bdellovibrio strains (9); S^{in} mutants of *Bdellovibrio* UK are different and exhibit a longer period of rapid motility before losing their flagella (2, 14). The latter would be expected to compete better with the S^d parent.

Although the *comp*$^-$ mutants of *Bdellovibrio* 109J (99.99% of their cells) are incapable of plaque formation, they attach (albeit less efficiently than the wild type) to *E. coli* cells and develop in their periplasmic space. They thus resemble the "H-I derivatives" isolated by Seidler and Starr (9), which failed to produce single plaques though they were bacteriolytic at high concentrations. The explanation suggested

by Seidler and Starr, that the absence of plaques is due to the dilution of certain factors present in the broth culture, does not hold in our case, since *comp*⁻ bdellovibrios attached to and lysed *E. coli* cells after being washed in buffer. Another possible explanation for the difference in the bacteriolytic capacity of the *comp*⁻ mutants in suspension and on plates could be that the establishment of the symbiotic relationship on a plate is dependent to a greater degree on efficient, active motility of the bdellovibrios than it is in a suspension. It is possible that S^{ln}*comp*⁺ mutants, which grow more slowly than S^{ln}*comp*⁻ mutants, also retain their flagella longer, and consequently attach better and form plaques. If this is true, then the difference between *comp*⁺ and *comp*⁻ mutants would be merely quantitative.

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