NOTES

Improved Cultivation Systems for Isolation of the Colorado Potato Beetle Spiroplasma

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In North America, the Colorado potato beetle, *Leptinotarsa decemlineata*, is often infected with the host-specific, gut-inhabiting Colorado potato beetle spiroplasma (CPBS). CPBS is apparently a commensal, but it may be useful in biocontrol if it can be transformed to express an insect-lethal gene. Difficulty in cultivating the organism, however, has hindered the development of a suitable transformation system. In this study, we eliminated the need for coculturing CPBS with insect cells. CPBS was reliably isolated with the BBL Anaerobic GasPak Jar system (low redox, enhanced CO₂), which was easier to use and less expensive than insect cell coculture methods. A further advantage is a reduction in contaminating insect cell components. Use of anaerobiosis should facilitate early-passage screening of isolates for extrachromosomal elements, for use in gene vector constructs. The unique spiral (decreasing amplitude of coils) morphology of CPBS was preserved by anaerobiosis. The use of low-pH (6.0 to 6.5) media allowed aerobic adaptation of CPBS to M1D and SP-4 broth media. These formulations permitted the first cultivation of CPBS on solid media, an accomplishment that will simplify the selection of molecular transformants. Potato beetles collected at four sites in Poland yielded CPBS strains similar to those previously obtained from populations in North America.

The Colorado potato beetle, *Leptinotarsa decemlineata*, a major pest of solanaceous crops, has few effective natural enemies. One promising biocontrol candidate is the Colorado potato beetle spiroplasma (CPBS) (*Prokaryotae*: class *Mollicutes*), a gut-inhabiting commensal specific to the genus *Leptinotarsa* (7). This spiroplasma is a potential candidate for genetic engineering to express toxins for biocontrol (5, 12). To accomplish this, strains of CPBS are needed from a wide geographical distribution and host range. Isolates will be evaluated as possible sources of extrachromosomal elements for construction of genetic vectors and for their suitability for transformation.

Such efforts have been hindered by a lack of suitable cultivation systems. Historically, standard spiroplasma media, including M1D and SP-4 (14), have proven to be unsuitable for primary isolation of CPBS. CPBS was isolated only in the insect tissue culture medium DCCM supplemented with *Diabrotica* cells (9). The resulting cultures had low titers and adapted to cell-free media only after multiple passage (11). However, multiple passage can cause spiroplasmas to lose extrachromosomal DNA (2). Furthermore, CPBS isolates that adapted to grow without insect cell supplements still required the expensive, inconvenient DCCM (14) and, because the organism could not be grown on solid media, efforts to select transformants were impeded.

Overall, use of the coculture system has resulted in low efficiencies of isolation from infected *L. decemlineata* beetles (8.1% [5 isolates from 62 infected beetles]) (7). Isolates were obtained from beetles collected in Maryland (strain LD-1), North Carolina (isolate NC-1), Michigan (isolate Ves-1), New Mexico (isolate Mex-1), Arizona (isolate Ari-1), and Quebec (isolate Can-1) but were not obtained from infected beetles collected in Alberta, Maine, Massachusetts, Minnesota, New Jersey, New York, Utah, and Washington. Although infected beetles were observed in Germany, Ukraine, and Byelorussia, the spiroplasmas were not cultivable by standard spiroplasma culture methods (17). Two isolates (Tex-2 and Tex-3) were obtained from 15 infected *L. texana* adult beetles (isolation efficiency, 13.3%) collected in Texas (7).

The present study addressed these deficiencies, with the goal of developing more dependable, accessible systems for primary isolation and aerobic broth culture of CPBS and a solid medium for eventual use in CPBS molecular transformation studies. To establish the broad applicability of optimized methods, CPBS isolates were obtained by sampling potato beetle populations in Maryland and Poland (*L. decemlineata*) and Texas (*L. texana*).

Sources of CPBS. Four sources of CPBS were used for primary isolation. The first three were gut fluid expressed from (i) spiroplasma-infected *L. decemlineata* larvae and adults maintained on potato plants at an experimental field at the U.S. Department of Agriculture Agricultural Research Center, Beltsville, Md., during 1991; (ii) spiroplasma-infected *L. texana* larvae and adults collected on *Solanum elaeagnifolium* (silver-

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FIG. 1. CPBS. (A and B) Electron micrographs of CPBS in gut fluid, showing an end-on view of the spiral form (A) and strain LD-1 in DCCM broth culture, showing the absence of cell wall and the presence of unit cytoplasmic membrane (B). (C and D) Dark-field micrographs of strain LD-1, showing a large medusa-like aggregation of helical cells (with individual helices extending outward from the edge of the medusa) in aerobic DCCM broth culture (C) and colonies on MID agar (2.0% Noble agar [pH 6.25]) after 4 days of incubation at 30°C in an aerobic environment (D).

leaf nightshade) in Hidalgo County near Mission, Tex., in May 1992; and (iii) spiroplasma-infected *L. decemlineata* adults collected from potato plants at four field sites in Poland (Pszczyna/Tarnowskie Góry, Lublin, Katowice, and Olkusz) in August 1993. The fourth source was the multipassaged CPBS group XX representative strain LD-1 (ATCC 43213 [Fig. 1B]); it was used to test growth on solid media and in low-pH broths. CPBS identification was confirmed by the serological deformation test (27) and by dark-field microscopic observation of gut contents (4). This spiroplasma is unique because, in the beetle gut, it often exists in the form of two-dimensional nonmotile compressed coils (Fig. 1A); upon exposure to culture media under anaerobic conditions or with cocultured insect cells, these coils become three-dimensional motile spirals, with turns of increasing amplitude (4). Other species of spiroplasma are helical (turns of equal amplitude), and CPBS becomes helical rather than spiral when cultured aerobically in the absence of insect cells (11). The spiroplasma often forms large (medusa-like) clumps (Fig. 1C) in the insect gut or in culture. No other spiroplasmas have been observed or detected in the potato beetle gut.

Growth parameters. For primary isolation, the culture systems were optimized (in terms of isolation frequency and high maximum titer) with respect to (i) medium type (DCCM, M1D, or SP-4) and formulation (broth or solid), (ii) oxygen tension (aerobic, anaerobic hood, microaerophilic CampyPak Plus, anaerobic GasPak, or CO₂ GasPak system), (iii) pH (5.0 to 7.5), and (iv) temperature (26 or 30°C). Isolation of CPBS by coculture with insect cells in DCCM (9, 10) was used as a standard for comparison of new methods. For coculture, the insect cell line IPLB-DU182E (18) growing in 1 ml of DCCM broth in 24-well tissue culture plates (Falcon) was used. Because we initially encountered difficulty in reviving strain LD-1 from lyophilized cultures in DCCM broth with Rehatuin fetal bovine serum (InterGen Co., Purchase, N.Y.), SP-4 containing one of three different serum samples was tested on multipassaged strain LD-1 prior to isolation attempts; the sera used were Rehatuin fetal bovine serum at pH 6.25 and Gibco-BRL (Life Technologies, Inc., Gaithersburg, Md.) and Hyclone (Hyclone Laboratories, Inc., Logan, Utah) fetal bovine serum at pHs 6.25 and 7.3 each. Higher titers (10⁹ rather than 10⁸ cells per ml) were obtained in the Gibco-BRL serum lot, which was subsequently used at a concentration of 10% (vol/vol). (This information is provided to indicate the importance of serum lot, not serum source, in the isolation of fastidious spiroplasmas. Sera vary greatly in suitability from lot to lot and by application.)

Growth media included DCCM, which had been used in previous work (9, 10), and the standard spiroplasma media M1D and SP-4 (14). DCCM differs from M1D and SP-4 in having larger amounts of free amino acids (12.8 g/liter for DCCM; 6.6 g/liter for M1D; 0.5 g/liter for SP-4) but smaller amounts of peptides (7.6 g/liter for DCCM; 11.2 g/liter for M1D; 12.8 g/liter for SP-4) and serum (10% for DCCM; 17% for M1D and SP-4) (10, 14). DCCM was developed for insect cell culture, whereas M1D and SP-4 were developed for culture of plant- and arthropod-associated spiroplasmas. While broth medium is traditionally used to cultivate fastidious spiroplasmas (9, 13), solid medium is needed for isolation of transformed colonies. Solid formulations (containing 2.0% Noble agar) of M1D and SP-4 (14) were also evaluated.

To test the effect of atmospheric conditions, static cultures were maintained aerobically or anaerobically in tubes with loose caps in an anaerobic hood (Bactron IV anaerobic chamber; Anaerobe Systems, San Jose, Calif.) containing 5% H₂ and 95% N₂ or in BBL GasPak Jar systems (Becton Dickinson

and Co., Cockeysville, Md.) with the anaerobic GasPak system (4 to 7% CO₂, 18 to 35% H₂, <0.1% O₂, remainder N₂), the CO₂ GasPak system (5 to 10% CO₂, 0% H₂, 18 to 20% O₂, remainder N₂), or the microaerophilic CampyPak Plus system (5 to 10% CO₂, 2% H₂, 5 to 15% O₂, remainder N₂).

The medium type and atmospheric conditions were optimized by using *L. decemlineata* beetles. Gut fluid was sampled by pressing gently on the beetle thorax and collecting up to 25 µl of regurgitated material. This was diluted with 0.5 ml of DCCM broth and filtered through a 0.45-µm-pore-size membrane (Millex-HA; Millipore Corp.) to remove microbial contaminants (4). Cell wall-less spiroplasmas pass through the filters and are collected in the filtrate; other microorganisms are retained on the filter. Filtrate (100 µl) was inoculated into 0.9 ml of medium and incubated at 26 or 30°C aerobically, in an anaerobic hood, or in an anaerobic GasPak system. Initial titers were 10⁷ to 10⁸ spiroplasma cells per ml. Typically, inoculations were made into 5 to 10 tubes of each medium under each atmospheric condition; insect cell cultures were grown aerobically only. Beetle infection was confirmed and the initial titer in culture was determined by enumeration of spiroplasma cells under dark-field (×400) microscopic illumination (4). To sample beetle hemolymph, insects were immersed for 1.5 min in 0.1% (wt/vol) NaOCl and then washed in sterile H₂O. With the exception of cultures in the anaerobic hood (which remained at ambient temperature) and those in the temperature trial (30°C), all cultures were maintained at 26°C. Samples were taken for observation, and passages were made once or twice a week, depending on the spiroplasma titer. Phenol red and bromocresol purple were used as growth indicators.

Once the system was optimized, isolation of spiroplasmas from the gut contents of 10 potato beetle adults collected in Poland (3 beetles from Pszczyna/Tarnowskie Góry, 1 from Lublin, 4 from Katowice, and 2 from Olkusz) was attempted. Hemolymph samples were obtained from three infected beetles from Pszczyna/Tarnowskie Góry. Each extract was added to an individual tube of H-2 broth; H-2 medium is a hybrid between DCCM and M1D (13). The suspensions were filtered, and individual filtrates containing microscopically discernible spiroplasmas (i.e., 10 gut isolates) were inoculated into DCCM broth and incubated aerobically (10 tubes) or anaerobically (10 tubes); M1D broth (10 tubes) was inoculated and incubated anaerobically.

To determine the effect of pH on primary isolation under aerobic conditions, extracts from the gut fluid of 31 *L. texana* specimens (6 adults and 25 larvae) were pooled and samples were inoculated into two tubes each of DCCM, M1D, and SP-4 broth adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0, or 7.5 with NaOH. For positive controls, DCCM (pH 6.5), M1D (pH 7.5), and SP-4 (pH 7.5) broths were inoculated with the same extract and incubated in the anaerobic GasPak System and aerobically in insect cell cultures in DCCM (pH 6.35).

The growth of aerobically adapted strain LD-1 at incubation temperatures of 26 and 30°C on M1D and SP-4 broth was studied at pHs 6.0, 6.5, 7.0, and 7.2. Attempts were also made to grow strain LD-1, which had been multiply passaged in DCCM broth, on solid media. The organism was inoculated into SP-4 medium (pH 6.5) and passaged six additional times. At passage 2, the SP-4 culture was also inoculated into M1D (pH 6.0) and passaged five times. These SP-4 and M1D subcultures were then subjected to titer determination in SP-4 and M1D broth buffered at pH 6.0, 6.5, 7.0, or 7.2 and incubated at 26 and 30°C. SP-4 and M1D broth cultures were also inoculated onto solid SP-4 (pH 6.5) and M1D (pH 6.0).

In all trials, growth titers were estimated by enumeration of motile cells under dark-field microscopy. Doubling times were estimated from the exponential curve $N_t = 2^t N_0$ (N_t is the

number of cells at time $t + 1$, N_0 is the initial number of cells, and t is the time in days). The cell morphology of strain LD-1 from cultures in the logarithmic phase was monitored by dark-field microscopy ($\times 400$ to $\times 1,250$). Electron microscopic techniques for spiroplasmas have been described previously (26).

Anaerobic conditions for primary isolation. Our experiments demonstrated the first successful use of anaerobic conditions for primary isolation of CPBS, an inhabitant of the anaerobic potato beetle gut (4). Short, translationally motile, spiral forms were visible by dark-field microscopy in both anaerobic insect cell-free culture and aerobic insect cell coculture controls. The isolation efficiency for anaerobic GasPak culture in DCCM broth was 100% ($n = 29$), with maximum titers (passage 10) of 5×10^9 to 1×10^{10} cells per ml and doubling times of 28.5 h at passage 5 and 9.6 to 14.4 h at passage 10. The BBL anaerobic GasPak system also permitted the primary isolation of CPBS in M1D and SP-4 broth (isolation efficiency was 100% for each [$n = 7$ in SP-4 and $n = 17$ in M1D]). Maximum titers and doubling times were comparable to those in DCCM, thus eliminating the need to use the more complicated DCCM.

The isolation efficiency was 60% ($n = 17$) in coculture with insect cells, with a lower maximum titer of 10^9 cells per ml and similar doubling times at passage 10 with respect to the results of anaerobic culture (see above). Use of anaerobiosis therefore not only is technically easier than coculture for CPBS isolation but also may be more reliable and may result in higher spiroplasma titers. Furthermore, primary isolation in cell-free media, made possible here by the use of anaerobiosis, should facilitate future efforts to obtain extrachromosomal elements for construction of gene vectors. After repeated passage (25 to 75 times), isolates from coculture wells or insect cell-free anaerobic cultures became adapted to aerobic culture in cell-free DCCM. CPBS isolates that were adapted to aerobic conditions reached titers of 5×10^9 cells per ml with doubling times in DCCM of 12.4 h at 26°C and 7.2 h at 30°C and in M1D of 15.2 h at 26°C and 7.2 h at 30°C (15). For comparison, strain LD-1 reached a maximum titer of 1.2×10^9 cells per ml with a doubling time of 7.5 h at passage 100 in aerobic culture (10). Large clumps (diameters, 1 to 8 μm) of spiroplasma cells (medusae) were visible in broth cultures (Fig. 1C).

Use of the anaerobic GasPak system facilitated the isolation of CPBS from 100% ($n = 10$) of infected *L. decemlineata* beetles sampled in Poland. Isolation was accomplished in both DCCM broth and M1D broth, with maximum titers of 5×10^9 cells per ml at passage 10 and doubling times of 9.6 to 14.4 h. As in previous studies (4, 7), primary isolates could not be obtained in DCCM under aerobic conditions and were obtained only from the guts of infected beetles and never from the hemocoel. The isolates were cell wall-less (Fig. 1B), grew in medusae (Fig. 1C), and were serologically similar to those from *L. decemlineata* and *L. texana* beetles collected in North America. The incidence of infection (30%, $n = 33$) was typical of rates in North America (17%, $n = 212$ [7]).

Roles of pH and temperature in CPBS isolation, culture on solid media, and morphology. Cultivation of CPBS, which inhabits the low-pH (5.9 to 6.6) potato beetle gut (3), was favored by media of low pH. High-passage CPBS strain LD-1 could adapt to aerobic broth when grown at low pH in DCCM (pH 6.35), M1D (pH 6.0 to 7.0), or SP-4 (pH 6.25 to 6.5). This strain was also cultured aerobically and anaerobically on solid media of low pH (SP-4, pH 6.5; M1D, pH 6.0). Colonies on 2.0% M1D agar were small (100 to 150 μm in diameter), with numerous satellite colonies established by migrating spiroplasmas (Fig. 1D). Since anaerobic GasPak system jars contain 4 to 7% CO_2 as a result of production of carbonic acid (21, 25), the

pH of SP-4 and M1D in these systems decreased (from 7.5 to 6.9) during spiroplasma growth. Nevertheless, provision of low pH does not, in itself, explain the success of the coculture and anaerobic systems, because primary isolation of CPBS under aerobic conditions was not accomplished over the pH range of 5.0 to 7.5 in DCCM or M1D.

Other studies (15) have shown that at pH 6.5, CPBS strain LD-1 grew to temperatures as high as 41°C in M1D but only to 37°C in DCCM, with a lower growth limit at 20°C and an optimum temperature of 30°C in both media. In the present study, this strain adapted to M1D up to pH 7.0 when cultured at 26°C but not at 30°C. At pH 6.0, the maximum titers were higher in M1D at 30°C ($>10^9$ cells per ml) than at 26°C (10^7 cells per ml). We suspect that interactions among temperature, pH, and medium are similar to that between temperature and the nutritional need for sphingomyelin reported for *Spiroplasma mirum* (6). At elevated temperatures, sphingomyelin was shown to be needed for membrane stability.

Growth conditions affected morphology and physiology as well as survival and growth titer. Spiroplasmas growing in DCCM with insect cells (which cause, among other effects, reduced oxygen tensions) or in insect cell-free broth under anaerobic conditions were small (three to four turns), translationally and flexionally motile spirals (Fig. 1A). In contrast, spiroplasmas growing under aerobic conditions in insect cell-free broths were long (10 to 12 turns) and helical (helical spiroplasma cells can be seen extending from the medusa in Fig. 1C) and lacked translational but not flexional motility.

Roles of O_2 , CO_2 , and H_2 in isolation. Although there is evidence of the presence of O_2 radical-protective superoxide dismutase and catalase in all mollicutes (20), and although other spiroplasmas are readily isolated under aerobic conditions (4), CPBS appears to be sensitive to O_2 , at least during primary isolation. However, not all reduced-oxygen or microaerophilic systems were equally suitable (Table 1). While the anaerobic GasPak system ($<0.1\%$ O_2) provided adequate conditions, the CO_2 GasPak and microaerophilic CampyPak systems did not, presumably because of their higher oxygen levels (18 to 20% and 5 to 15%, respectively).

Since the anaerobic hood ($<0.1\%$ O_2) was also not adequate, we suspect that in addition to O_2 , other factors, such as enhanced CO_2 (4 to 7%) or H_2 (18 to 35%) levels, may be important as well. The fact that primary isolates of CPBS can be obtained in insect cell coculture sheds little light on which of these two gases is important, because insect cells (in addition to lowering the O_2 level) produce both CO_2 and H_2 .

CO_2 is reported to have important beneficial (23, 24) or deleterious (1) effects on microbes, including mollicutes (16, 21). While levels of 4 to 10% CO_2 are beneficial for some anaerobes (23) and fastidious strains of *Spiroplasma kunkelii* (16), *Mycoplasma hyorhinis*, a microaerophile that prefers 5% O_2 and that does not tolerate redox levels less than -115 mV, is inhibited by 5% CO_2 (21). Presumably, these mollicutes may use O_2 to reoxidize NADH (22). Mollicute fixation of CO_2 via the pyruvate carboxylase-catalyzed pathway from pyruvate to oxaloacetate has been reported (19).

Nevertheless, although an enhanced CO_2 level has often been used in anaerobic systems and a level of 5% (but generally not 2.5 or 7.5%) CO_2 has been shown to increase the growth titers of many anaerobes (24), no one has shown enhanced CO_2 levels to be necessary for mollicute isolation, except in the context of lowering the O_2 concentration.

Recommended procedure for CPBS culture. The recommended procedure is as follows. Surface sterilize potato beetles by immersion in 0.1% (wt/vol) NaOCl, wash them in sterile H_2O , and blot dry. Gently press the thorax of the beetle

TABLE 1. Effect of culture conditions on primary isolation and adaptation of CPBS

Effect and system ^a	Atmosphere (%)				Medium	pH ^b	mOs of the medium	Temp (°C)
	CO ₂	H ₂	O ₂	N ₂				
Primary isolates of CPBS obtained								
Coculture			21	79	DCCM	6.35→5.1	375	
Anaerobic GasPak	4–7	18–35	<0.1	Re ^c	DCCM	6.35	375	
Anaerobic GasPak	4–7	18–35	<0.1	Re	SP-4	7.5	360	
Anaerobic GasPak	4–7	18–35	<0.1	Re	M1D	7.5→5.5	520	
Primary isolates of CPBS not obtained								
CO ₂ GasPak	5–10	0	18–20	Re	DCCM	6.25	375	
Microaerophilic	5–10	2	5–15	Re	DCCM	6.25	375	
Anaerobic hood	0–0.3	5	<0.1	95	DCCM	6.25	375	
Aerobic broth	0.3	0	21	79	DCCM	5.0–7.5	375	
Aerobic broth	0.3	0	21	79	SP-4	5.0–7.5	360	
Aerobic broth	0.3	0	21	79	M1D	5.0–7.5	520	
CPBS isolates adapted								
Aerobic broth	0.3	0	21	79	DCCM	6.25	375	
Aerobic broth	0.3	0	21	79	SP-4	6.25–6.5	360	26
						≠7.0 or 7.2		
Aerobic broth	0.3	0	21	79	SP-4	6.5	360	30
						≠7.0 or 7.2		
Aerobic 0.8 or 2.5%	Solid (agar)		21	79	SP-4	6.5		
Aerobic broth	0.3	0	21	79	M1D	6.0–7.0	520	26
						≠7.2		
Aerobic broth	0.3	0	21	79	M1D	6.0	520	30
						≠7.0 or 7.2		
Aerobic 0.8 or 2.5%	Solid (agar)		21	79	M1D	6.0		
CPBS isolates did not adapt								
CO ₂ GasPak	5–10	0	18–20	Re	DCCM	6.25	375	
Microaerophilic	5–10	2	5–15	Re	DCCM	6.25	375	
Anaerobic hood	0–0.3	5	<0.1	95	DCCM	6.25	375	
Aerobic broth	0.3	0	21	79	SP-4	7.0–7.5	360	
Aerobic broth	0.3	0	21	79	M1D	7.2–7.5	520	

^a BBL anaerobic jar systems included the anaerobic GasPak system, the CO₂ GasPak system, and the microaerophilic CampyPak Plus system. The anaerobic hood was a Bactron IV anaerobic chamber (Anaerobe Systems).

^b Values preceded by “≠” indicate that CPBS could not be cultivated at those pHs.

^c Re, residual.

to cause it to regurgitate 5 to 25 μ l of gut fluid into a microcapillary tube, and inoculate this fluid into 0.5 ml of medium (DCCM, SP-4, or M1D). Pass this suspension through a 0.45- μ m-pore-size filter to remove contaminants. Inoculate 100 μ l of filtrate into 0.9 ml of medium (DCCM [pH 6.25 to 6.5], SP-4 [pH 6.5 to 7.5], or M1D [pH 6.5 to 7.5]), and incubate at 26 to 30°C in a BBL anaerobic GasPak system (4 to 7% CO₂, 18 to 35% H₂, <0.1% O₂, remainder N₂). Monitor the cultures by dark-field microscopy ($\times 400$ to $\times 1,250$) for the presence of motile cells, and confirm the identity of spiroplasmas by serologic testing (spiroplasma motility inhibition [8] or deformation [27] tests). Passages should be done at 3- to 7-day intervals or whenever the culture achieves a 10-fold increase in titer; also, titers above 10⁹ cells per ml should be avoided because cells in stationary phase are entering senescence. After multiple (10 to 100) 10-fold anaerobic passages, attempts can be made to adapt the spiroplasma to grow aerobically in DCCM, SP-4, or M1D at pH 6.25 to 6.5. Monitor daily for titer increases (titers may initially decrease to below 5 $\times$ 10⁶ cells per ml while the spiroplasma is adapting to aerobic conditions). For isolation of extrachromosomal elements, it is advisable to utilize early-passage CPBS in anaerobic culture. Also, although spiroplasmas used in transformation could conceivably be early-passage isolates cultured under anaerobic conditions, a well-adapted late-passage strain (i.e., one that has been passaged multiple times aerobically or anaerobically) is probably preferable. In either case, an SP-4 or M1D solid medium (2% agar) at pH 6.5 can be used.

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