# Grouping of Seven Biologically Defined Isolates of Cucumber Mosaic Virus by Peptide Mapping

Michael C. Edwards and Dennis Gonsalves

Former graduate research assistant and associate professor, Department of Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva 14456.
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## ABSTRACT

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Coat proteins of seven biologically defined isolates of cucumber mosaic virus were compared by peptide mapping and enzyme-linked immunosorbent assay (ELISA). All seven isolates could be classified in either of two subgroups, confirming previous reports of the existence of these groups. Isolates B, C-1, C-2, and F were placed in one subgroup, while isolates L-2, L-3, and WL were placed in the other. Isolates within a subgroup were indistinguishable. One-dimensional peptide mapping appears to be a reliable and sensitive technique that can complement existing viral classification techniques.

Cucumber mosaic virus (CMV) is a multicomponent, aphid-borne virus of worldwide importance. In New York, pathogenicity and virulence of CMV isolates vary considerably (9,18,19), which complicates control of diseases incited by CMV.

Recently, we investigated the genetics of CMV in relation to host resistance using two naturally occurring CMV isolates (B and L-2) (5). CMV-L-2 (previously LsS) infects *Lactuca saligna* L. (Plant Introduction [PI] 261653), a lettuce breeding line resistant to most CMV isolates, but does not infect beans or most other legumes (19). CMV-B, on the other hand, infects legumes but not *L. saligna* L. (18). We showed that both L-2 RNAs 2 and 3 are necessary for pathogenicity of L-2 to *L. saligna* L. Although this indicates that B RNA 3 and L-2 RNA 3 differ significantly, coat proteins of these two strains were indistinguishable by immunodiffusion in agar gels (5). However, immunodiffusion cannot detect all potential differences between the coat proteins of the two isolates.

Thus, one objective of this work was to compare these proteins using the peptide mapping technique of Cleveland et al (2). This technique can detect internal differences other than the antigenic differences that are revealed by immunodiffusion. A second objective was to determine whether our biologically distinct isolates could be differentiated into groups.

In this report, we show that CMV-B and CMV-L-2 coat proteins are distinct, although serologically related. We also show that both ELISA and peptide mapping may be useful for classifying CMV isolates.

## MATERIALS AND METHODS

**Virus isolates.** All isolates have been differentiated on the basis of host resistance (Table 1) and were originally isolated in New York, except CMV-F, which was isolated in France. Both CMV-F and CMV-B were isolated from bean (*Phaseolus vulgaris* L.). CMV-C-1 was isolated from squash (*Cucurbita pepo* L.), while CMV-C-2 was isolated from an $F_1$ hybrid of *Cucurbita pepo* L. × *Cucurbita martinezii* Bailey. Only CMV-C-2 can infect *C. martinezii* Bailey. Both CMV-L-2 and CMV-L-3 were isolated from lettuce (*Lactuca sativa* L.) and can infect *L. saligna* (PI 261653). CMV-L-2 induces no symptoms in *L. serriola* L. (ACC 500-4), while CMV-L-3 causes systemic necrosis in the same host. CMV-WL was isolated from tomato (*Lycopersicon esculentum* Mill.), in which it induces "white leaf" symptoms as a result of its association with a low molecular weight RNA 5 (9). This isolate is also pathogenic to *L. saligna* L.

**Virus propagation and purification.** All isolates were propagated in *Cucurbita pepo* 'President' (Zucchini-type squash). Virus-infected tissue was harvested 10–12 days after inoculation and processed essentially as described by Lot et al (15). After the final high-speed centrifugation, virus pellets were suspended in PE buffer (0.01 M $NaH_2PO_4$, 0.001 M EDTA, pH 7.0) containing 0.001 M $NaN_3$.

Virus preparations for immunization were centrifuged in 10–40% linear sucrose gradients for 3 hr at 23,000 rpm in a Beckman SW 25.1 rotor. Virus bands were drawn off using a syringe and sucrose was removed by dialysis overnight against PE buffer. Virus was then concentrated through high-speed centrifugation (3 hr at $28 \times 10^3$ rpm in a Beckman 30 rotor) and resuspended in PE buffer at a concentration of 1 or 2 mg/ml. Virus was then fixed with 0.2% formaldehyde (6).

**Immunization and ELISA procedures.** Antisera to isolates B, C-1, L-2, and WL were produced by intramuscular injection of New Zealand white rabbits with purified virus emulsified 1:1 with Freund's adjuvant. Virus (2 mg) was first injected with complete adjuvant and subsequently with incomplete adjuvant (1 mg at weekly intervals). Bleeding at weekly intervals began 3 wk after the first injection. ELISA tests were done according to Clark and Adams (1). Absorbance at 405 mm was measured with a Dynatech model 2-580 ELISA reader.

**Coat protein isolation.** Coat protein and RNA were separated by boiling a 1:1 (v/v) mixture of purified virus and degradation buffer (0.01 M $NaPO_4$ (pH 7.0), 2% SDS, 2% mercaptoethanol, and 20% sucrose) for 2 min, followed by discontinuous polyacrylamide slab gel electrophoresis (100 V, constant voltage, ~5 hr). Running buffer and gels (5% stacking gel, 12% separating gel) were prepared as described by Laemmli (13).

Our method for recovering protein from gels was based upon the procedures of Hager and Burgess (10). The coat protein band was visualized by soaking the gel in cold 0.25 M KCl for 4–5 min. The band was then sliced from the gel, rinsed in cold distilled water for 15 min, and frozen until use. For eluting protein, gel pieces were thawed, sliced about 1 mm thick, and placed in elution buffer (0.05 M tris-HCl (pH 7.9), 0.1% SDS, 0.1 mm EDTA, and 0.15 M NaCl). After stirring slowly at room temperature for several hours, liquid was drawn off and the protein was precipitated by adding trichloroacetic acid with a final concentration of 20% and centrifuging at 10,000 rpm for 10 min in a Sorvall SS34 rotor. The pellet was rinsed twice with ether and resuspended in digestion buffer (0.125 M tris-HCl (pH 6.8), 0.5% SDS, and 10% glycerol) (2).

**Peptide mapping.** Coat proteins were compared using essentially

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the technique of Cleveland et al (2). Initially, conditions were standardized so that we obtained the same band patterns for albumin digests as did Cleveland. All coat protein samples (0.25 mg/ml final concentration) to be compared using a particular enzyme were digested simultaneously and under identical conditions. Chymotrypsin and papain (C-3142 and P-4762, respectively; Sigma Chemical Co., St. Louis, MO 63178) were used at a 1:10 enzyme to substrate ratio, whereas *Staphylococcus aureus* V8 protease (39-900-1; Miles Laboratories, Elkhart, IN) was used at a 1:5 ratio. Enzyme-substrate mixtures were incubated at 37 C for either 0.5 (papain), 1 (V8 protease), or 24 hr (chymotrypsin). Protein digests were electrophoresed on discontinuous (5% stacking, 15% separating) polyacrylamide minislab gels (Idea Scientific Co., Corvallis, OR 97339) at 200 V (constant voltage) for ~1 hr. Gels and buffers were prepared according to Laemmli (13). Gels were stained with Coomassie blue R-250 (0.3% in 50% methanol/10% acetic acid) for several hours, followed by destaining in 30% methanol/10% acetic acid.

## RESULTS

**Peptide mapping.** Limited digestion of the coat proteins of various CMV isolates with V8 protease, chymotrypsin, and papain produced the band patterns shown in Fig. 1. Identical patterns were observed in three other experiments. Proteins of isolates B, F, C-1, and C-2 were indistinguishable from each other, but strikingly different from isolates L-2, L-3, and WL. Conversely, isolates L-2, L-3, and WL were indistinguishable from each other, yet easily distinguished from isolates B, F, C-1, and C-2.

Digestions with V8 protease (Fig. 1A) and chymotrypsin (Fig. 1B) were much less complete than those with papain (Fig. 1C), although differences in band patterns were much more obvious. Coat proteins of isolates B, C-1, C-2, and F were more completely digested by V8 protease than were coat proteins of isolates L-2, L-3, and WL. Band patterns of digests of the latter showed only one major band with several more faint, lower molecular weight minor bands. Electrophoresis of C-1, C-2, B, and F digests produced band patterns with five major bands, only one of which comigrated with a band in the corresponding patterns of the other isolates.

Digestion of isolates C-1, C-2, B, and F with chymotrypsin produced six major products, whereas digestion of isolates L-2, L-3, and WL produced five major products. Most noticeably, the fourth and fifth bands in patterns from digested C-1, C-2, B, and F coat proteins were absent in patterns from digested L-2, L-3, and WL coat proteins.

Papain, the least specific enzyme of those tested, gave the most complete digestion (Fig. 1C). Band patterns for all seven isolates were very similar, but not identical. A band was present just below the coat protein band in digest patterns of isolates C-1, C-2, B, and F, but not in digest patterns of isolates L-2, L-3, and WL. Bands representing the lowest molecular weight products of the L-2, L-3, and WL digests were absent in the patterns of the C-1, C-2, B, and F digests. The double band just below the papain band was most easily visible in the C-2 digest, but is evident in digests of isolates B, C-1, and F. It is also barely detectable in digests of isolates L-2, L-3, and WL.

While the molecular weights of the various coat proteins were not calculated, coat proteins of isolates L-2, L-3, and WL migrated more slowly than those of isolates B, C-1, C-2, and F.

**ELISA tests.** In three separate tests, comparisons of four (B, C-1,

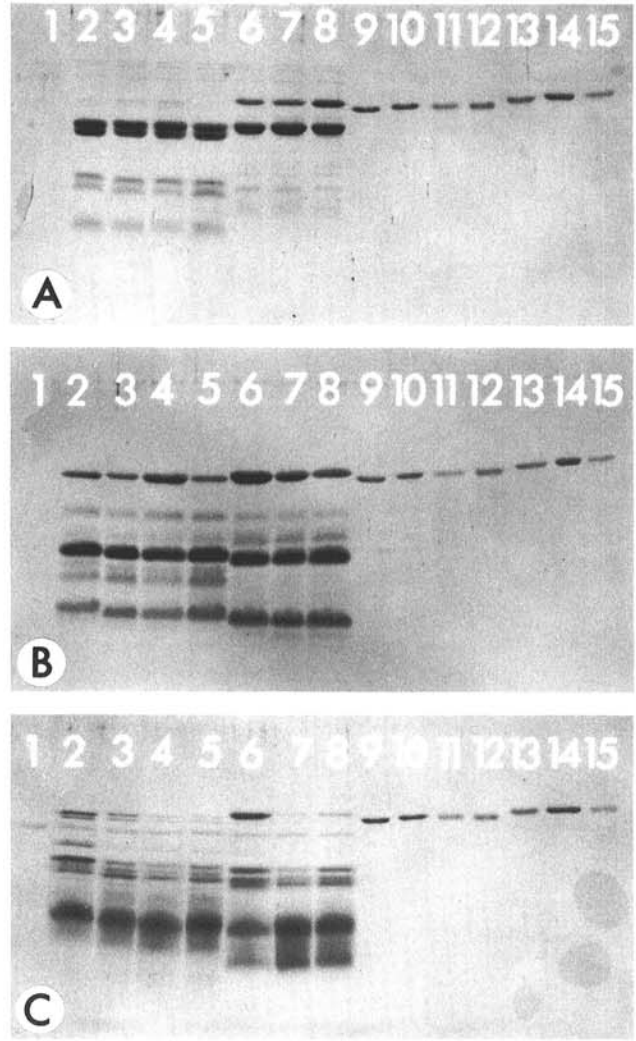


**Fig. 1.** Patterns of peptide bands produced after electrophoresis of partial enzymatic digests of coat proteins of seven cucumber mosaic virus isolates. **A,** Electrophoresis of partial V8 protease digests. Well 1 was loaded with 0.1 μg V8 protease. Wells 2–8 were loaded with 0.5 μg each of digested protein from isolates C-2, F, C-1, B, L-2, L-3, and WL, respectively. Wells 9–15 were loaded with 0.1 μg each of undigested coat protein from isolates C-2, F, C-1, B, L-2, L-3, and WL, respectively. **B,** Electrophoresis of partial chymotrypsin digests. Wells were loaded as in 1A except that 1 μg of digested material was used. **C,** Electrophoresis of partial papain digests. Wells were loaded as in 1B.

TABLE 1. Differentiation of cucumber mosaic virus isolates by host resistance and symptomatology

| Strain | Host species | | | | | |
|---|---|---|---|---|---|---|
| | *Cucurbita pepo* | *Cucurbita martinezii* | *Phaseolus vulgaris* | *Lactuca saligna* PI 261653 | *Lactuca serriola* ACC 500-4 | *Lycopersicon esculentum* |
| B | +[a] | − | + | − | − | LD, Mo |
| C-1 | + | − | − | − | − | LD, Mo |
| C-2 | + | + | − | − | − | LD, Mo |
| F | + | − | + | − | − | LD, Mo |
| L-2 | + | − | − | + | S | LC |
| L-3 | + | − | − | + | N | LC |
| WL | + | − | − | + | NT | LC (WL:RNA 5)[b] |

[a] + = susceptible; − = resistant; LC = leaf curl; LD = leaf distortion; Mo = mosaic; N = necrosis; S = symptomless; WL = white leaf; and NT = not tested.
[b] CMV-WL RNA 1 + 2 + 3 induce LC, while CMV-WL RNAs 1 + 2 + 3 + 5 induce WL.

L-2, WL) of the seven isolates using ELISA supported the peptide mapping analysis (Fig. 2). Using antisera to all four isolates, we distinguished two serological groups. All antisera reacted most strongly with virus isolates from the homologous isolate's group.

Antisera to isolates C-1 and L-2 were quite specific and clearly differentiated each of the groups (Fig. 2A and D). On the other hand, antisera to isolates B and WL were less able to distinguish between homologous and nonhomologous antigens (Fig. 2B and C).

## DISCUSSION

Figures 1 and 2 clearly indicate that each of our isolates may be classified into either one of two groups. Several techniques have been used previously to differentiate and classify CMV isolates. Initially, two groups were defined serologically based on immunodiffusion in agar gels (3,4). These groups correlated with groups defined by host range tests (16). Physical and chemical properties (14), hybridization with cDNA (8), and competition hybridization (17) have also been used to establish two major groups. While most of the isolates in our study differ from those used previously by others, we feel that our groups correlate with previously defined groups. Piazzolla et al (17) used three New York isolates in their competition hybridization study. One of these, NYB, is the equivalent of our B isolate. All three New York isolates were placed in the WT group (the DTL serogroup of Devergne and Cardin [3]). Thus, our B, C-1, and C-2, and F isolates fit into this group as well. We are uncertain whether the L-2, L-3, and WL isolates should be placed in the same group or the S group of Piazzolla (17) [ToRS serogroup (3)], since completely different isolates were used.

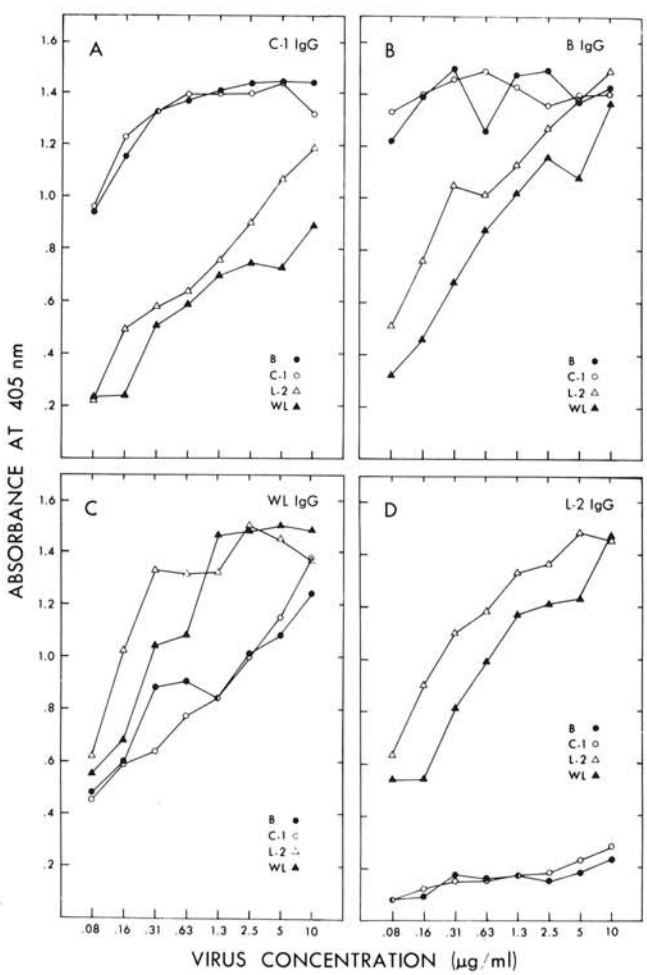


**Fig. 2.** Reactions of CMV isolates B, C-1, L-2, and WL in ELISA tests using both homologous and heterologous antisera. Antigens (purified virus) were diluted serially. Each point represents the mean of two readings. **A,** C-1 antiserum; **B,** B antiserum; **C,** WL antiserum; **D,** L-2 antiserum.

There is some indication that the L-2, L-3, and WL isolates are members of the S group. Their proteins migrate slower than those of the B, C-1, C-2, and F isolates. This correlates well with the differences in amino acid content between proteins of isolates in the two major CMV groups (11). Not only do amino acid contents of the coat proteins of isolates S and D differ significantly, but also CMV-S coat protein is slightly larger (235 amino acids) than CMV-D coat protein (229 amino acids) (11). More extensive comparison of the two groups seems warranted to see if protein size is a useful taxonomic parameter.

Recently, good correlation was found between serological typing and peptide mapping of tymovirus proteins (12). As with CMV here, some isolates that were indistinguishable serologically were indistinguishable by the peptide mapping technique of Cleveland et al (2).

This one-dimensional mapping technique is relatively simple to use and it has exceptional resolving power. Classification through peptide mapping is a sensitive and consistent means of supplementing symptomatological or serological classification. CARNA 5 or satellite RNAs can affect symptomatological tests (9). We were unable to differentiate any of the isolates used in this study by immunodiffusion in agar gels (5).

While peptide maps were reproducible, ELISA results were conclusive with only two of our four antisera. Two serological groups were distinguished, but specificity of the antisera varied. This variation may simply reflect differences between individual rabbits used to produce the antiserum. Immunization was not a significant factor since schedules were the same in each case.

Hybridization techniques have the advantage of dealing with the complete genome, but are more cumbersome and expensive than one-dimensional peptide mapping. As pointed out by Cleveland et al (2), mapping requires no expensive reagents or equipment and resolution can be expanded by using additional enzymes. While digestion conditions should be optimized, band patterns do not change over a fairly broad range of enzyme concentrations or digestion times (2). Minigels (83 × 103 × 0.8 mm) gave sharper band patterns and required much less sample than larger gels. Silver staining would increase sensitivity still further. Minigels also decreased running, staining, and destaining times. Hence, peptide mapping appears to be a useful means to define CMV subgroups.

Our results show that the coat proteins of CMV isolates B and L-2 are distinct. The significance of this in relation to known pathogenicity differences cannot yet be assessed. Lot and Kaper (14) suggested that CMV coat protein may influence the expression of RNA 3. Coat protein of certain tripartite genome viruses activates replication (7). It is possible that the 34-kdalton protein encoded by RNA 3 (20) or even a noncoding region of RNA 3 influences host specificity as with infection of *Lactuca saligna* by CMV-L-2.

## LITERATURE CITED


1. Clark, M. F., and Adams, A. M. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
2. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252:1102-1106.
3. Devergne, J. C., and Cardin, L. 1973. Contribution à l'étude du virus de la mosaïque du concombre (CMV). IV. Essai de classification de plusieurs isolats sur la base de leur structure antigénique. Ann. Phytopathol. 5:409-430.
4. Devergne, J. C., and Cardin, L. 1975. Relations sérologiques entre cucumovirus (CMV, TAV, PSV). Ann. Phytopathol. 7:255-276.
5. Edwards, M. C., Gonsalves, D., and Provvidenti, R. 1983. Genetic analysis of cucumber mosaic virus in relation to host resistance: Location of determinants for pathogenicity to certain legumes and *Lactuca saligna*. Phytopathology 73:269-273.
6. Francki, R. I. B., and Habili, N. 1972. Stabilization of capsid structure and enhancement of immunogenicity of cucumber mosaic virus (Q

strain) by formaldehyde. Virology 48:309-315.
7. Fulton, R. W. 1981. Ilarviruses. Pages 377-413 in: E. Kurstak, ed. Plant Virus Infections and Comparative Diagnosis. Elsevier/North-Holland Biomedical Press, New York.
8. Gonda, T. J., and Symons, R. H. 1978. The use of hybridization analysis with complementary DNA to determine the RNA sequence homology between isolates of plant viruses: Its application to several isolates of cucumoviruses. Virology 88:361-370.
9. Gonsalves, D., Provvidenti, R., and Edwards, M. C. 1982. Tomato white leaf: The relation of an apparent satellite RNA and cucumber mosaic virus. Phytopathology 72:1533-1538.
10. Hager, D. A., and Burgess, R. R. 1980. Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: Results with sigma subunit of *Escherichia coli* RNA polymerase, wheat germ DNA topoisomerase, and other enzymes. Anal. Biochem. 109:76-86.
11. Kaper, J. M., and Waterworth, H. E. 1981. Cucumoviruses. Pages 257-332 in: Plant Virus Infections and Comparative Diagnosis. E. Kurstak, ed. Elsevier/North-Holland Biomedical Press, New York.
12. Koenig, R., Francksen, H., and Stegemann, H. 1981. Comparison of tymovirus capsid proteins in SDS-polyacrylamide-porosity gradient gels after partial cleavage with different proteases. Phytopathol. Z. 100:347-355.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.
14. Lot, H., and Kaper, J. M. 1976. Physical and chemical differentiation of three isolates of cucumber mosaic virus and peanut stunt virus. Virology 74:209-222.
15. Lot, H., Marrou, J., Quiot, J. B., and Esvan, C. 1972. Contribution à l'étude du virus de la mosaïque du concombre (CMV). I. Méthode de purification rapide du virus. Ann. Phytopathol. 4:25-38.
16. Marrou, J., Quiot, J. B., and Marchoux, G. 1975. Caractèrisation de douze souches du VMC par leurs aptitudes pathogènes: Tentative de classification. Meded. Fac. Landbouwwet. Rijks. Univ. Gent. 40:107-122.
17. Piazzolla, P., Diaz-Ruiz, J. R., and Kaper, J. M. 1979. Nucleic acid homologies of eighteen cucumber mosaic virus isolates determined by competition hybridization. J. Gen. Virol. 45:361-369.
18. Provvidenti, R. 1976. Reaction of *Phaseolus* and *Macroptilium* species to a strain of cucumber mosaic virus. Plant Dis. Rep. 60:289-293.
19. Provvidenti, R., Robinson, R. W., and Shail, J. W. 1980. A source of resistance to cucumber mosaic virus in *Lactuca saligna* L. for lettuce (*L. sativa* L.). HortScience 15:528-589.
20. Schwinghamer, M. W., and Symons, R. H. 1977. Translation of the four major RNA species of cucumber mosaic virus in plant and animal cell free systems and in toad oocytes. Virology 79:88-108.