A *Chrysodeixis chalcites* Single-Nucleocapsid Nucleopolyhedrovirus Population from the Canary Islands Is Genotypically Structured To Maximize Survival

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A *Chrysodeixis chalcites* single-nucleocapsid nucleopolyhedrovirus wild-type isolate from the Canary Islands, Spain, named ChchSNPV-TF1 (ChchTF1-wt), appears to have great potential as the basis for a biological insecticide for control of the pest. An improved understanding of the genotypic structure of this wild-type strain population should facilitate the selection of genotypes for inclusion in a bioinsecticidal product. Eight genetically distinct genotypes were cloned *in vitro*: ChchTF1-A to ChchTF1-H. Quantitative real-time PCR (qPCR) analysis confirmed that ChchTF1-A accounted for 36% of the genotypes in the wild-type population. In bioassays, ChchTF1-wt occlusion bodies (OBs) were significantly more pathogenic than any of the component single-genotype OBs, indicating that genotype interactions were likely responsible for the pathogenicity phenotype of wild-type OBs. However, the wild-type population was slower killing and produced higher OB yields than any of the single genotypes alone. These results strongly suggested that the ChchTF1-wt population is structured to maximize its transmission efficiency. Experimental OB mixtures and cooccluded genotype mixtures containing the most abundant and the rarest genotypes, at frequencies similar to those at which they were isolated, revealed a mutualistic interaction that restored the pathogenicity of OBs. In OB and cooccluded mixtures containing only the most abundant genotypes, ChchTF1-ABC, OB pathogenicity was even greater than that of wild-type OBs. The ChchTF1-ABC cooccluded mixture killed larvae 33 h faster than the wild-type population and remained genotypically and biologically stable throughout five successive passages *in vivo*. In conclusion, the ChchTF1-ABC mixture shows great potential as the active ingredient of a bioinsecticide to control *C. chalcites* in the Canary Islands.

Baculoviruses are highly effective control agents for a number of lepidopteran pests due to their host specificity and outstanding safety record (1). Dozens of baculoviruses are currently produced on a commercial scale and applied to large areas of crops, such as the nucleopolyhedrovirus (NPV) of *Anticarsia gemmatalis* (AgMNPV) in Brazil (2), the NPV of *Helicoverpa armigera* in China (3), or the granulovirus of *Cydia pomonella* in Europe (4). Considerable genetic heterogeneity is usually observed in natural baculovirus populations (5). However, wild-type baculoviruses, which can be collected as natural isolates, are not patentable, and genotypic characterization is required to select the genotype or mixture of genotypes with the insecticidal characteristics that favor their use as biological control agents.

This diversity has been demonstrated by the characterization of different geographical isolates of the same virus (6, 7) and also within single isolates, which frequently comprise a mixture of genotypes (8, 9, 10). In wild-type populations, genetic diversity seems to be an important aspect of virus survival under field conditions to allow adaptation to varied environmental conditions (11, 12). Genotypic variability is commonly determined with the application of standard molecular tools, such as restriction endonuclease analysis (REN), PCR (5, 7, 8), or, more recently, denaturing gradient gel electrophoresis (DGGE) (13). Studies on genotypic heterogeneity within baculovirus populations using *in vitro* (14, 15) or *in vivo* (8, 9, 16) techniques, or bacterial artificial chromosomes (BACs) (17), have revealed that intraspecific variability is due to genomic reorganizations, DNA deletions or insertions, recombination, or mutations (8).

Infections with experimental mixtures of different genotypes of a given viral species or different viral species have shown antagonistic or synergistic effects in the phenotypic traits resulting from these interactions (18, 19, 20). Such effects may include the modification of biological parameters, such as pathogenicity, speed of kill, or virus yield. In some instances, the mixture of viral genotypes enhances the efficiency of the virus as a biological insecticide (21, 22, 23), but contrasting results have also been reported in other virus-host systems (24, 25). Interest in baculoviruses as biological insecticides has led to the search for effective isolates comprising either single genotypes or genotype mixtures, making genotypic characterization of field isolates a key step in the process of active-material selection during bioinsecticide development (5, 6).

Chrysodeixis chalcites (Lepidoptera: Noctuidae) is a major polyphagous pest in many countries (26, 27). In the Canary Islands, Spain, *C. chalcites* larvae can cause up to 30% production loss in banana crops (28). To date, different geographical *C. chalcites* single-nucleocapsid nucleopolyhedrovirus (ChchSNPV) strains have been described: one from the Netherlands (ChchSNPV-NL) (27, 29); another from Almería, Spain (ChchNPV-SP1) (30); and five from Tenerife, Canary Islands, Spain (ChchSNPV-TF1 to -TF5) (31). The ChchSNPV-TF1 isolate was recently selected for further development as a bioinsecticide for protection of banana crops due to its high pathogenicity and speed of kill against an insect population from the

Canary Islands (31). The aims of the present study were, first, to evaluate the genetic structure of the ChchSNPV-TF1 wild-type isolate and, second, to determine the outcome of genotype interactions for the insecticidal phenotype of genotype mixtures by testing different occlusion body (OB) and coocluded mixtures. Finally, the genetic and biological stability of the most insecticidal genotypic mixture was evaluated through five serial passages *in vivo*.

MATERIALS AND METHODS

Insect, viruses, and cells. *C. chalcites* larvae were obtained from pupae received from the Instituto Canario de Investigaciones Agrarias (ICIA), Tenerife, Spain, in 2007 and refreshed periodically with pupae from the Canary Islands. Previous quantitative real-time PCR (qPCR) studies indicated that the insect colony did not harbor an inapparent nucleopolyhedrovirus infection (31). Larvae were reared at $25 \pm 1°C$ and $70\% \pm 5\%$ humidity with a photoperiod of 16:8 (light-dark) on a semisynthetic diet described by Greene et al. (32). Adults were fed 30% (wt/vol) diluted honey. ChchSNPV-TF1 (abbreviated here as ChchTF1-wt) was originally isolated from a single larva collected during a natural epizootic in banana crops in Tenerife, Canary Islands, Spain. ChchTF1-wt OBs were amplified once in fifth-instar (L_5) *C. chalcites* larvae from the laboratory colony. The BTI-TN-5B1-4 *Trichoplusia ni* cell line, also known as High Five Cells (Invitrogen, Life Technologies Ltd., Carlsbad, CA, USA), was maintained in TNM-FH insect medium (Gibco, Life Technologies Ltd.) supplemented with 10% fetal bovine serum (FBS) (Gibco).

***In vitro* virus cloning and restriction endonuclease analysis.** For the isolation of individual genotypes, L_5 larvae that had been inoculated with a 90% lethal concentration (LC_{90}) (5×10^7 OBs ml^{-1}) of ChchTF1-wt OBs were surface decontaminated with 70% ethanol, and a hemolymph sample was obtained by bleeding at 48 h postinfection (p.i.). Each hemolymph sample was filtered through a 0.45-μm filter, serially diluted in TNM-FH medium, and used to infect 2×10^6 High Five cells as previously described (15). After 10 days, clearly separated plaques were picked individually with a sterile Pasteur pipette and transferred to a vial containing 0.5 ml phosphate-buffered saline (PBS). A 100-μl volume of each plaque-PBS suspension was amplified in 5×10^5 cells per well in six-well tissue culture plates. Ten days after infection, the medium and cells were collected and centrifuged ($3,800 \times g$; 5 min) to pellet the cells, whereas the supernatant, containing the budded virions (BVs), was stored at 4°C. For clone amplification, 5 μl of these suspensions was injected into five L_5 *C. chalcites* larvae, which were then reared individually on the diet described above. Mortality was recorded daily. Virus-killed larvae were individually transferred to a microcentrifuge tube and stored at −20°C until required.

OBs of the different clones were purified from dead larvae, and DNA was purified as previously described (31). For restriction endonuclease analysis, viral DNA was mixed with BglII, as the enzyme allowed clear discrimination between the different ChchSNPV isolates (31).

Biological activity of single ChchTF1 genotypes. The insecticidal characteristics of the isolated genotypes were assessed in terms of mean lethal concentration (LC_{50}), mean time to death (MTD), and OB production (OBs larva^{-1}), as an expression of pathogenicity, virulence, and OB yield, respectively, in *per os* insect bioassays (33).

To determine OB pathogenicity, second-instar *C. chalcites* larvae (L_2) were starved for 8 to 12 h at 26°C and then allowed to drink from an aqueous suspension containing 10% (wt/vol) sucrose, 0.001% (wt/vol) Fluorella blue, and OBs at one of the following concentrations: 1×10^5, 2×10^4, 4×10^3, 8×10^2, and 1.6×10^2 OBs ml^{-1}. This range of concentrations was previously determined to kill between 95 and 5% of the experimental insects (31, 34). Bioassays with 25 larvae per virus concentration and 25 larvae as negative controls were performed three times. Mortality results were subjected to logit analysis using the POLO-PC program (35).

To determine speed of kill, time-mortality analyses were performed in groups of 25 L_2 *C. chalcites* larvae inoculated orally with OB concentrations that resulted in ~90% larval mortality: 3.31×10^4

OBs ml^{-1} for ChchTF1-wt and 2.22×10^5, 2.33×10^5, 3.01×10^5, 2.36×10^5, 2.91×10^5, 1.60×10^5, 1.90×10^5, and 1.87×10^5 OBs ml^{-1} for genotypes ChchTF1-A, ChchTF1-B, ChchTF1-C, ChchTF1-D, ChchTF1-E, ChchTF1-F, ChchTF1-G, and ChchTF1-H, respectively. Larval mortality was recorded every 8 h until the insects had either died or pupated. The whole bioassay was carried out three times. Time-mortality data were subjected to Weibull survival analysis using the Generalized Linear Interactive Modeling (GLIM) program (36).

Finally, infected larvae from the speed-of-kill bioassay were used to determine OB production from at least 20 larvae for each virus treatment in each replicate (total, ~60 larvae per virus treatment). Each larva was thawed and individually homogenized in 1 ml distilled water, and the total OB yield per larva was estimated by counting in triplicate in a Neubauer hemocytometer. OB yields were averaged for each replicate and subjected to analysis of variance using the SPSS v12 program (SPSS Inc., Chicago, IL).

Construction and biological activity of OB and coocluded mixtures. Four OB mixtures were prepared using *in vitro*-purified OBs of the different genotypes. These OBs were mixed in proportions similar to those at which they were isolated: 58% ChchTF1-A plus 42% ChchTF1-B (ChchTF1-AB); 47% ChchTF1-A plus 34% ChchTF1-B plus 19% ChchTF1-C (ChchTF1-ABC); 47% ChchTF1-A plus 34% ChchTF1-B plus 18% ChchTF1-C plus 1% ChchTF1-G (ChchTF1-ABCG); 46% ChchTF1-A plus 33% ChchTF1-B plus 19% ChchTF1-C plus 1% ChchTF1-G plus 1% ChchTF1-H (ChchTF1-ABCGH).

To produce coocluded genotype mixtures, the OBs of each genotype were mixed in the desired proportions (identical to the OB mixtures), and fifth-instar larvae were orally inoculated with these mixtures (5×10^7 OBs ml^{-1}) by the droplet feeding method. OBs were purified from infected larvae, and these OBs were considered coocluded genotype mixtures. The coocclusion of genotype mixtures was confirmed as described below.

To confirm the relative frequencies of the different genotypes in the coocluded mixtures and in the wild-type population, a qPCR was performed using genotype-specific primers. To design these primers, five genomic regions of ChchTF1 were sequenced. These regions were highly variable, according to the ChchSNPV-NL sequence information (GenBank accession number AY864330; 29) and other reports (37, 38). These regions were outside the complete coding regions of *hoar* (*chch4*; nucleotides [nt] 2,965 to 6,504 in the ChchSNPV-NL genome), *bro-a* (*chch55*; nt 48,823 to 50,376), *bro-b* (*chc69*; nt 68,861 to 66954), *bro-c* (*chch70*; nt 70,060 to 69,254), and *bro-d* (*chc114*; nt 114,806 to 113,517) (Table 1). PCR products were amplified using high-fidelity *Taq* polymerase (PrimeStar; TaKaRa Bio Inc., Shiga, Japan), purified using the QIAquick PCR Purification Kit (Qiagen, Düsseldorf, Germany), and used for direct sequencing (Sistemas Genómicos S.L., Valencia, Spain). The recent availability of the complete sequences of ChchTF1-A, -B, and -C confirmed that the most variable regions in these genomes are the *hoar* and *bro-d* genes. Multiple-sequence alignments were carried out with Clone Manager version 9 (Scientific & Educational Software, Cary, NC, USA).

Once sequences had been analyzed, different sets of primers were designed in the most variable region. Nontemplate controls (NTCs) were analyzed for each set of primers in order to verify the absence of nonspecific background signal. Milli-Q water was used in all reactions as a negative control. Primer sets for each genotype (Table 1) were selected based on the presence of a single melting peak, a measure of specific amplification. A 1:100 dilution of DNA (1 μl) extracted from 1×10^8 OBs of the different mixtures was used for qPCR. Wild-type genomic DNA was also included in the assay. All qPCRs were performed using SYBR green Premix *Ex Taq* (Tli RNaseH Plus; TaKaRa Bio Inc., Shiga, Japan). A 1-μl volume of CsCl-purified DNA of single genotypes was quantified using a UV spectrophotometer (Eppendorf AG, Hamburg, Germany), and serial dilutions (10^{-1} to 10^{-8} ng μl^{-1}) were used as internal standards for each qPCR. The identity of each genotype was previously confirmed by restriction endonuclease analysis using BglII. All reactions were performed under the following conditions: 2 min at 50°C; 30 s at 95°C; 45 cycles at 95°C

TABLE 1 Specific PCR primers designed for the amplification of complete *hoar*, *bro-a*, *bro-b*, *bro-c*, and *bro-d* genes and qPCR primers designed for the quantification of the relative frequencies of the different ChchTF1-wt genotypes

Primer	Sequence (5′–3′)	Position in ChchSNPV-NL genome	PCR/qPCR fragment size (bp)[a]						Amplification purpose
			NL	A	B	C	G	H	
hoar.F	TTATTCTATTTTTTGAACTG	2965–2984	3,540	3,636	3,804	3,900	3,540	3,396	Primers used to amplify the complete *hoar* gene
hoar.R	ATGATACGGAAATTTAAATC	6504–6485							
bro-a.F	ATGTCTCTCTCAAAAATAAATC	48823–48844	1,554	1,554	1,554	1,557	1,554	1,557	Primers used to amplify the complete *bro-a* gene
bro-a.R	TTATTTTCTGCCCGACGCTTTG	50355–50376							
bro-b.F	TTATTCGTTAGAACTTTTATTG	66954–66975	1,908	1,887	1,887	1,908	1,908	1,908	Primers used to amplify the complete *bro-b* gene
bro-b.R	ATGACTACATCCAAAATCATG	68861–68841							
bro-c.F	CTACATAATGTTGCAGTTGAG	69254–69274	807	807	807	807	807	807	Primers used to amplify the complete *bro-c* gene
bro-c.R	ATGAATTCTAAAGTCATTACC	70060–70040							
bro-d.F	TTATTCTATAGCCAAGGTATTTG	113517–113539	1,290	1,611	828	420	828	420	Primers used to amplify the complete *bro-d* gene
bro-d.R	ATGGATTATCGCCCCCTTGAAAC	114806–114784							
Abro-d.F	CGAGTATTTACCAGTTTTCC	114462–114481		217					Primers used to quantify the relative frequency of ChchTF1-A in the OB and cooccluded mixtures by qPCR
Abro-d.R	GATGGATTATCGCCCCCTTG	114678–114659							
Cbro-d.F	GGAATCCAGTTTTCCTCAATTC	114881–114902				212			Primers used to quantify the relative frequency of ChchTF1-C in the OB and cooccluded mixtures by qPCR
Cbro-d.R	CCTAAATCAGGACCCCCTTG	115092–115073							
DNA-pol.F	TTAATGCGTTCGCTGCTCAC	54174–54193	91	91	91	91	91	91	Primers used to quantify the relative frequency of all genotypes in the OB and cooccluded mixtures by qPCR
DNA-pol.R	ATCACGCCTTTTCCAAGTGC	54264–54245							

[a] NL, ChchSNPV from The Netherlands (29); A, B, C, G, H, ChchTF1-A, -B, -C, -G, and -H.

for 5 s, 65°C for 30 s, and 95°C for 15 s; 60°C for 15 s; and 95°C for 15 s for the dissociation curve. The qPCR results of each replicate were subjected to analysis of variance using the SPSS v12 program. All reactions were performed in duplicate, and the experiment was repeated three times.

To verify the cooclusion of the different genotypes within a single OB, a qPCR analysis was performed with 100 L_2 larvae orally inoculated with 1.6×10^2 OBs ml^{-1} of the ChchTF1-ABC cooccluded mixture. This dilution was expected to kill ~5% of the inoculated larvae. Previous studies indicated that L_2 larvae drank an average of 0.41 μl of suspension (A. Bernal, unpublished data), that is, ~0.26 OBs larva^{-1} at the OB concentration used, which meant that almost all infections in virus-killed larvae occurred following the ingestion of a single OB. As such, qPCR detection of different genotypes from single larvae was considered to be a reliable indicator of their cooclusion in mixtures within a single OB. The experiment was performed five times. A 1:100 dilution of DNA (1 μl) extracted from OBs purified from a single larva was subjected to qPCR analysis as described previously.

Finally, the insecticidal activities of the OB mixtures and cooccluded genotype mixtures were compared with those of ChchTF1-wt OBs and the individual genotype OBs used to produce the mixtures. The pathogenicity, speed of kill, and OB productivity were determined in L_2 as described previously. For wild-type and individual genotypes, the OB concentrations used for these assays were those used in the single-genotype assays described above. For OB mixtures and cooccluded mixtures, inoculum concentrations to determine pathogenicity were the same as those described above, whereas the following concentrations were used for speed-of-kill and OB productivity assays: 4.37×10^4, 3.49×10^4, 4.22×10^4, and 4.37×10^4 OBs ml^{-1} for the four OB mixtures (ChchTF1-AB, ChchTF1-ABC, ChchTF1-ABCG, and ChchTF1ABCGH, respectively), and 5.31×10^4, 3.73×10^4, 4.91×10^4, and 5.19×10^4 OBs ml^{-1} for the cooccluded mixtures (ChchTF1-AB, ChchTF1-ABC, ChchTF1-ABCG, and ChchTF1-ABCGH, respectively).

Serial passage *in vivo* of a cooccluded genotype mixture. A serial-passage experiment was performed three times with the selected mixture (ChchTF1-ABC) and ChchTF1-wt. A group of 50 *C. chalcites* L_5 larvae were inoculated *per os* with the corresponding LC_{90} of each inoculum and individually reared on the diet described above until death or pupation. Virus-killed cadavers were collected and pooled in 50-ml plastic centrifuge tubes, purified, and used to infect the next batch of experimental larvae. The four subsequent passages were initiated with the same number of larvae and OB concentration to complete five passages. The relative frequencies of the different genotypes from each passage were analyzed by qPCR as described above. Finally, the pathogenicities of ChchTF1-ABC and the wild type were also determined for the OBs from the final passage and compared with that of OBs from the first passage, as previously described.

RESULTS

Genotypic structure of ChchTF1-wt. A total of 117 ChchTF1 clones were amplified in cell culture. The plaques they produced in *T. ni* cells were small, and all clones rendered similarly low replication titers compared with the plaques and replication titers produced by the type baculovirus, *Autographa californica* nucleopolyhedrovirus (AcMNPV), in the same cells. Eight different genotypes were identified, named ChchTF1-A to ChchTF1-H based on differences in their BglII restriction profiles (Fig. 1). The ChchTF1-A restriction profile was indistinguishable from that of ChchTF1-wt and was the most frequently observed, representing 36% (*n* = 42) of the plaque-purified clones. ChchTF1-A, together with ChchTF1-B (26%) and ChchTF1-C (14%), accounted for the majority (76%) of isolated clones. The other genotypes were pres-
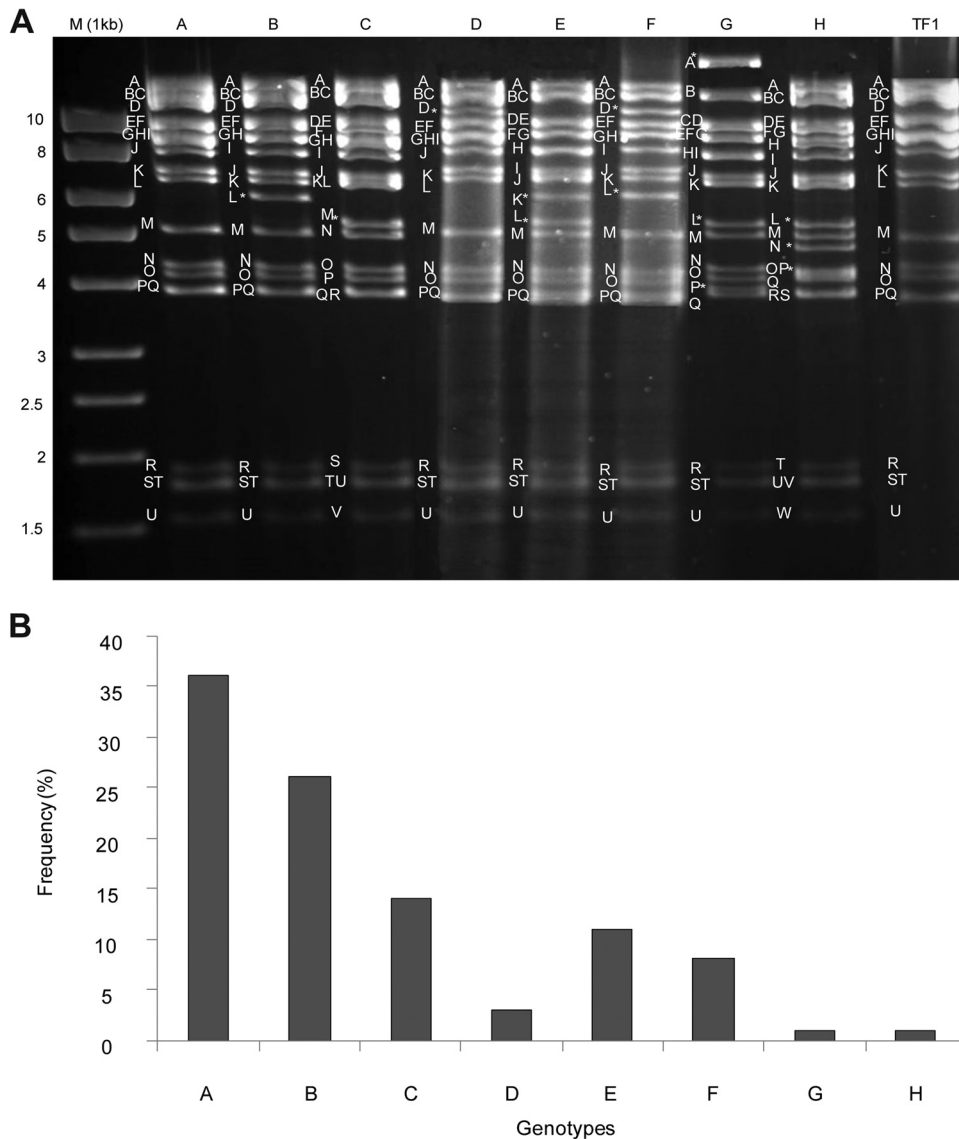
FIG 1 (A) Restriction endonuclease profiles of the DNAs of ChchTF1-wt (TF1) and its cloned genotypes ChchTF1-A to ChchTF1-H digested with BglII. The fragments were named alphabetically, with the letter A given to the largest BglII fragment. The 1-kb DNA Marker Ladder (Stratagene) was used as a molecular size marker (kbp) (lane M). The asterisks mark RFLPs. (B) Frequencies at which the different genotypes were cloned.

ent at lower frequencies—ChchTF1-F (11%), ChchTF1-E (8%) and ChchTF1-D (3%)—whereas ChchTF1-G and ChchTF1-H each originated from a single clone (~1%).

All genotypes showed restriction fragment length polymorphisms (RFLPs) with respect to ChchTF1-wt or ChchTF1-A. ChchTF1-B, -C, and -D genotypes each showed a unique profile involving the marker fragments BglII-L, BglII-M, and BglII-D, respectively. Genotypes ChchTF1-E and ChchTF1-F could be differentiated by two polymorphisms involving BglII-K and BglII-L, and BglII-D and BglII-L, respectively. Finally, the restriction profiles of ChchTF1-G and ChchTF1-H showed three polymorphisms in BglII-A; BglII-L and BglII-P; and BglII-L, BglII-N, and BglII-P, respectively. No submolar bands were observed in these genotypes, and the restriction profiles remained invariant for at least three passages in insects.

Biological activities of individual ChchTF1 genotypes. The

LC_{50} of ChchTF1-wt was estimated at 1.61×10^3 OBs ml^{-1}, and it was 5.6- to 11.1-fold more pathogenic than any of its component genotypes in single infections (Table 2). The broadly overlapping 95% fiducial limits of the relative potency values of individual genotypes indicated that the OBs of these genotypes did not differ significantly in their insecticidal potencies.

Analysis of MTD values revealed that the wild-type population and the cloned genotypes differed significantly in speed of kill (Table 2); the genotypes ChchTF1-B, ChchTF1-C, ChchTF1-D, and ChchTF1-E were significantly faster killing (~11 to 16 h faster) than the wild type, which was the slowest-killing (131 h p.i.) treatment tested (Table 2).

Mean OB production values were normally distributed. OB yields per larva differed significantly between genotypes ($F_{(8,18)}$ [the first number meaning the intergroup degrees of freedom and the second meaning the within-group degrees of freedom] =

TABLE 2 LC_{50}, relative potency, and MTD values of ChchTF1-wt and its individual genotypes, ChchTF1-A to ChchTF1-H, in second-instar *C. chalcites*[a]

Virus inoculum	LC_{50} (OBs ml^{-1})	Relative potency	Fiducial limit (95%)		MTD (h)	Fiducial limit (95%)	
			Low	High		Low	High
ChchTF1-wt	1.61×10^3	1			131	126	136
ChchTF1-A	1.27×10^4	0.12	0.08	0.19	125	122	128
ChchTF1-B	8.87×10^3	0.18	0.11	0.28	117	115	119
ChchTF1-C	8.92×10^3	0.18	0.11	0.28	115	113	117
ChchTF1-D	1.03×10^4	0.15	0.10	0.24	120	118	122
ChchTF1-E	1.69×10^4	0.09	0.06	0.14	119	117	121
ChchTF1-F	1.39×10^4	0.11	0.07	0.17	126	121	129
ChchTF1-G	1.69×10^4	0.09	0.06	0.15	133	131	137
ChchTF1-H	1.35×10^4	0.11	0.07	0.18	129	127	131

[a] Logit regressions were fitted in POLO Plus (35). A test for nonparallelism was not significant for the treatments ($\chi^2 = 4.52$; df = 6; $P > 0.05$). Relative potencies were calculated as the ratio of effective concentrations relative to ChchTF1. MTD values were estimated by Weibull survival analysis (36), using the fitted hazard function (α [shape parameter] = 6.5329).

9.730; $P < 0.001$). ChchTF1-wt was the most productive (7.80×10^6 OBs/larva), whereas single genotypes yielded an average of 7.22×10^6 to 5.23×10^6 OBs larva^{-1} (Fig. 2). Only the ChchTF1-A genotype was as productive as the wild-type strain (Tukey; $P = 0.952$), while the other single genotypes were ~1.5-fold less productive than ChchTF1-wt. Interestingly, ChchTF1-B, ChchTF1-C, ChchTF1-D, and ChchTF1-E were as productive as the other single genotypes, despite their higher speed of kill (Fig. 2). In contrast, ChchTF1-F, ChchTF1-G, and ChchTF1-H, which were as fast killing as the wild-type population, were among the least productive genotypes.

Genomic variability between ChchTF1 genotypes. Sequencing of the variable genomic regions of the ChchTF1 genotypes revealed greater variability in the *hoar* and *bro-d* genes, whereas *bro-a*, *bro-b* and *bro-c* were less variable and had 99 to 100% homology to those of ChchSNPV-NL. Recently, the complete genome sequences of ChchTF1-A (accession number JX535500), ChchTF1-B (JX560539), and ChchTF1-C (JX560540) genotypes were determined and confirmed that the most variable regions within these viruses were the *hoar* and *bro-d* genes. In the *hoar* gene, all genotypes showed deletions or insertions, so that *hoar* gene lengths differed in all genotypes compared to that of ChchSNPV-NL (Table 1). ChchTF1-A, ChchTF1-B, and ChchTF1-C had insertions of 96, 264, and 360 nt, respectively. ChchTF1-G had no deletions or insertions, whereas ChchTF1-H had a 144-nt deletion compared to ChchSNPV-NL (Table 1). As a result, ChchTF1-A to ChchTF1-G were 96, 92, 87, 99, and 94% homologous to the ChchSNPV-NL *hoar* gene, respectively. The *bro-d* genes of ChchTF1 single genotypes also presented variations with respect

to that of ChchSNPV-NL: a 321-nt insertion in ChchTF1-A, a 462-nt deletion in ChchTF1-B and ChchTF1-G, and 870-bp deletions in ChchTF1-C and ChchTF1-H. Sequence homology to ChchSNPV-NL varied from 80% for ChchTF1-A to 38% for ChchTF1-B and ChchTF1-G to just 21% for ChchTF1-C and ChchTF1-H (Table 1). This gene was selected for primer design, as it allowed differentiation of genotypes.

Genotype abundance in OB mixtures and coccluded mixtures. For the OB mixtures, genotypes were mixed at frequencies similar to those at which they were isolated *in vitro*: ChchTF1-AB (58%:42%), ChchTF1-ABC (47%:34%:19%), ChchTF1-ABCG (47%:34%:18%:1%), and finally ChchTF1-ABCGH (46%:33%: 19%:1%:1%). OB progeny obtained following oral inoculation of larvae with these OB mixtures were confirmed to be coccluded mixtures.

Of the different sets of primers designed in the *bro-d* gene, only those that differentiated ChchTF1-A and ChchTF1-C could be used in the qPCR. This was mostly due to the fact that all primers designed in the *bro-d* variable region of the other genotypes produced more than one melting peak in the qPCRs or dimers and lacked specificity. Due to the unavailability of specific primers for ChchTF1-B and the other genotypes, a set of primers common to all genotypes was designed in the *DNA-polymerase* gene (Table 1). Therefore, the relative proportion of ChchTF1-B was estimated by the difference between the amplification obtained for the *DNA-polymerase* gene common to all genotypes (100%) and those obtained specifically for ChchTF1-A and -C.

Analysis by qPCR confirmed that the genotype ratio in the OB mixtures reflected the frequency of isolation in tissue culture from the original inocula. In the ChchTF1-AB mixture, the two genotypes were present at prevalences of 58% and 42%, respectively, closely reflecting their abundance in the plaque-purified clones (36% A:26% B, for a total of 62%), i.e., 58% of 62 is 36, and 42% of 62 is 26. In the ChchTF1-ABC mixture, the frequencies of the three genotypes were estimated at 47%, 38%, and 14%, respectively, which also resembled their cloning frequency *in vitro* (36% A:26% B:14% C, for a total of 76%), i.e., 47% of 76 is 36, 38% of 76 is 29, and 14% of 76 is 11. In the ChchTF1-ABCG mixture, the frequency of ChchTF1-A was estimated at 47%, the frequency of ChchTF1-B plus -G was estimated at 40%, and the frequency of ChchTF1-C was estimated at 13%. The 2% increase in the *DNA-polymerase* amplification with respect to that in mix-
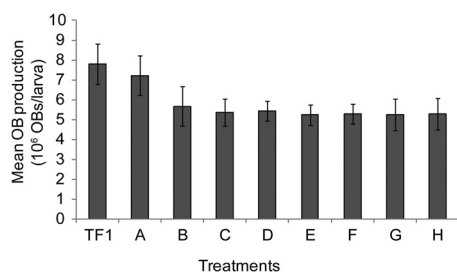
FIG 2 Mean OB yields obtained from infection of *C. chalcites* second-instar larvae at LC_{90} with ChchTF1-wt (TF1), ChchTF1-A (A), ChchTF1-B (B), ChchTF1-C (C), ChchTF1-D (D), ChchTF1-E (E), ChchTF1-F (F), ChchTF1-G (G), and ChchTF1-H (H). The error bars indicate standard errors.

TABLE 3 LC_{50}, relative potency, and MTD values of ChchTF1-wt; its individual genotypes ChchTF1-A, -B, -C, -G, and -H; and OB and cooccluded mixtures of cloned genotypes ChchTF1-AB, -ABC, -ABCG, and -ABCGH in second-instar *C. chalcites*[a]

Virus inoculum	LC_{50} (OBs ml^{-1})	Relative potency	Fiducial limit (95%)		MTD (h)	Fiducial limit (95%)	
			Low	High		Low	High
Wild type							
ChchTF1-wt	1.34×10^3	1			126	121	131
Individual genotype							
ChchTF1-A	9.14×10^3	0.19	0.08	0.26	123	120	126
ChchTF1-B	5.24×10^3	0.26	0.14	0.46	116	114	118
ChchTF1-C	1.12×10^4	0.12	0.07	0.21	113	109	117
ChchTF1-G	1.73×10^4	0.08	0.04	0.15	127	121	133
ChchTF1-H	9.42×10^3	0.21	0.12	0.37	125	120	130
OB mixtures							
ChchTF1-AB	1.34×10^3	1.01	0.56	1.80	115	110	120
ChchTF1-ABC	5.75×10^2	2.34	1.17	4.70	112	105	119
ChchTF1-ABCG	9.09×10^2	1.48	0.83	2.66	113	106	120
ChchTF1-ABCGH	1.96×10^3	0.69	0.40	1.19	111	104	120
Cooccluded mixtures							
ChchTF1-AB	2.13×10^3	0.63	0.36	1.10	105	97	113
ChchTF1-ABC	6.44×10^2	2.09	1.06	4.13	93	90	96
ChchTF1-ABCG	1.93×10^3	0.70	0.40	1.22	99	96	102
ChchTF1-ABCGH	1.77×10^3	0.76	0.42	1.35	98	95	101

[a] Logit regressions were fitted in POLO Plus (35). A test for nonparallelism was not significant for the treatments ($\chi^2 = 12.71$; df = 13; $P = 0.470$). Relative potencies were calculated as the ratio of effective concentrations relative to ChchTF1. MTD values were estimated by Weibull survival analysis (36), using the fitted hazard function (α [shape parameter] = 8.3674).

ture ChchTF1-ABC was attributed to the addition of ChchTF1-G to the initial inoculum at a frequency of 1%, although in this case, no direct estimate could be performed due to lack of suitable specific primers. As such, these frequencies closely matched the genotype prevalence in the plaque-purified clones (36% A:26% B:14% C:1% G, for a total of 77%). Similarly in the ChchTF1-ABCGH mixture, the frequency of ChchTF1-A was estimated at 46%, ChchTF1-B plus -G plus -H at 41%, and ChchTF1-C at 13%. A 1% increase in the *DNA-polymerase* amplification signal with respect to that in the mixture ChchTF1-ABCG was attributed to the presence of ChchTF1-H at a frequency of 1%. As a result, the genotype frequencies in this mixture closely mimicked the frequencies estimated from *in vitro* cloning (36% A:28% B:14% C:1% G:1% H, for a total of 78%). Similarly, several ChchTF1-wt samples were analyzed, and the prevalences of ChchTF1-A and ChchTF1-C were estimated at 34% A:12% C, which was also similar to estimates generated from *in vitro* cloning, whereas 54% of the amplifications reflected the presence of the remaining genotypes.

Coocclusion of different genotypes in a single OB was also confirmed by qPCR. In total, 23 larvae died after having consumed a single OB; both ChchTF1-A and ChchTF1-C were successfully amplified from all OB samples taken from each cadaver, indicating that both genotypes had replicated in these insects and were present in OBs. The frequencies of the three genotypes after infection (49% A:34% B:17% C) were similar to that at which they were inoculated (47%:34%:19%).

Biological activities of different OB and cooccluded genotypic mixtures. Dose-response bioassays revealed that the pathogenicity values of ChchTF1-AB, ChchTF1-ABCG, and ChchTF1-ABCGH OBs, in both OB mixtures and cooccluded mixtures, were

restored to that of the wild-type population or were ~2-fold lower (in ChchTF1-ABC as both OB and cooccluded mixtures) than the wild-type OBs (Table 3). Speed-of-kill analysis revealed three distinct groups: the fastest-killing group comprised the cooccluded mixtures ChchTF1-ABC, ChchTF1-ABCG, and ChchTF1-ABCGH, with MTD values of 93, 99, and 98 h p.i., respectively. The second group comprised ChchTF1-B; ChchTF1-C; ChchTF1-AB OBs and cooccluded mixtures; and ChchTF1-ABC, ChchTF1-ABCG, and ChchTF1-ABCGH OB mixtures, which had intermediate MTD values ranging from 105 to 116 h. Finally, ChchTF1-wt, ChchTF1-A, ChchTF1-G, and ChchTF1-H constituted the slowest-killing inocula (Table 3). Considering both parameters (OB pathogenicity and speed of kill), the ChchTF1-ABC cooccluded mixture was selected for the active material as the basis for a biological insecticide based on OB potency and virulence properties.

Significant differences in OB yields were observed in insects infected by the different inocula ($F_{(13,28)} = 20.755$; $P < 0.001$). ChchTF1-wt and ChchTF1-A were the most productive treatments, with 8.26×10^6 and 7.67×10^6 OBs larva^{-1}, respectively (Tukey; $P = 0.236$), followed by the ChchTF1-AB OB mixture, which was as productive as ChchTF1-A (Tukey; $P = 0.060$) but significantly less productive than ChchTF1-wt (Tukey; $P < 0.05$). The other single genotypes and mixtures were significantly less productive, with means ranging from 5.62×10^6 to 4.32×10^6 OBs larva^{-1} (Fig. 3).

Stability of the ChchTF1-ABC cooccluded mixture throughout serial passages. The relative frequencies of ChchTF1-A ($F_{(5,12)} = 2.453$; $P = 0.094$), ChchTF1-B ($F_{(5,12)} = 1.965$; $P = 0.157$), and ChchTF1-C ($F_{(5,12)} = 0.366$; $P = 0.862$) in the ChchTF1-ABC cooccluded mixture did not differ significantly over five successive passages. The average frequencies of these ge-
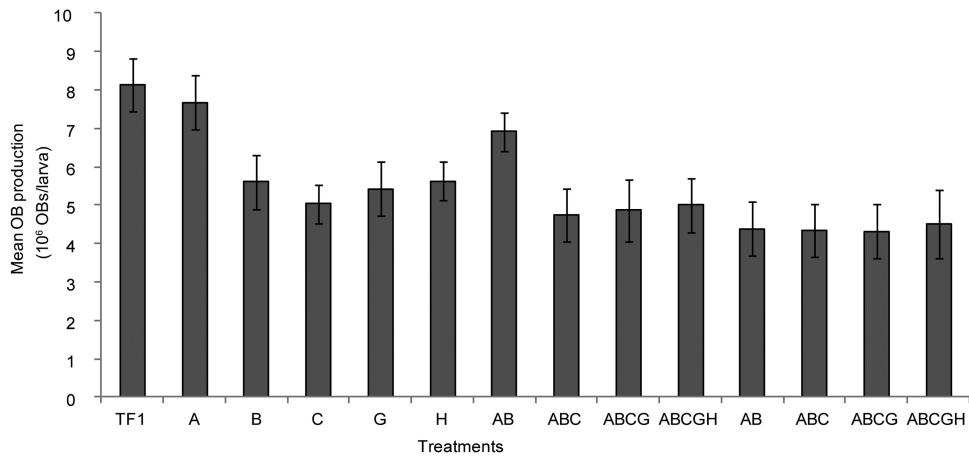
FIG 3 Mean OB yields obtained from infection of *C. chalcites* second-instar larvae at LC$_{90}$ with ChchTF1-wt (TF1); its individual genotypes ChchTF1-A, ChchTF1-B, ChchTF1-C, ChchTF1-G, and ChchTF1-H; the OB mixtures ChchTF1-AB, ChchTF1-ABC, ChchTF1-ABCG, and ChchTF1-ABCGH; and the cooccluded genotype mixtures ChchTF1-AB, ChchTF1-ABC, ChchTF1-ABCG, and ChchTF1-ABCGH. The error bars indicate standard errors.

notypes in the mixture were 48, 34, and 18%, respectively, compared with the starting frequencies, 36% A:26% B:14% C (Fig. 4A). The frequencies of ChchTF1-A ($F_{(5,12)} = 2.640$; $P = 0.078$) and ChchTF1-C ($F_{(5,12)} = 1.450$; $P = 0.276$) in the wild-type population also did not differ significantly over five successive passages, at 34% and 12%, respectively (Fig. 4B). This genetic stability was also reflected in the pathogenicity values of the corresponding mixed-genotype OBs. The LC$_{50}$s of ChchTF1-wt OBs and the ChchTF1-ABC cooccluded-mixture OBs at the fifth passage were 1.26×10^3 and 7.59×10^2 OBs ml^{-1}, respectively, which did not differ significantly from values estimated at the first passage (Table 3). Similarly, ChchTF1-ABC OBs from the fifth passage were 1.67-fold more potent than wild-type OBs from the same passage, as observed with the initial inocula (Table 2).

DISCUSSION

The genetic structure of the most prevalent and pathogenic isolate of ChchSNPV from the Canary Islands, ChchTF1-wt, and the interactions among its genotypes were examined with the aim of selecting a genotype or mixture of genotypes with valuable insecticidal traits. Eight different genotypes were identified in the ChchTF1-wt population. The BglII profile of the single genotype ChchTF1-A was the same as that of ChchTF1-wt, suggesting it is likely to be an abundant genotype in the natural population. The abundance of this genotype may have masked submolar bands from other, less frequent genotypes (31). The dominance of ChchTF1-A was confirmed by qPCR analysis and was present in ~36% of the plaque isolates from the wild-type population. The presence of a particular dominant genotype in nucleopolyhedrovirus populations has also been reported in *Spodoptera exigua* MNPV (16, 39) and *Spodoptera frugiperda* MNPV (15). Pure genotypes of ChchTF1-C, ChchTF1-G, and ChchTF1-H presented BglII profiles similar to those of previously described isolates: ChchSNPV-TF3, ChchSNPV-NL, and ChchSNPV-TF2, respectively (31). The fact that the ChchTF1-G genotype presented the same profile as the Dutch strain suggests a phylogenetic relationship between the Dutch and Canary Island populations and also underlines the importance of differences in the contribution of each genotype to the survival of nucleopolyhedroviruses in genetically distinct host populations and under different environmental conditions. Genome sequencing and phylogenetic, evolutionary and selection analyses would be necessary to support this hypothesis.

Phenotypic differences are derived from changes at the genome level (40, 41) and are important for virus adaptation and survival (8, 42). The major source of intraspecific variability among genotypes can be found in homologous-repeat regions (hrs) (5), the *bro* gene family (5, 11, 37), and the *hoar* gene (38). For ChchTF1 genotypes, the greatest variability was observed within the *hoar* and *bro-d* genes. Recently, the complete genome
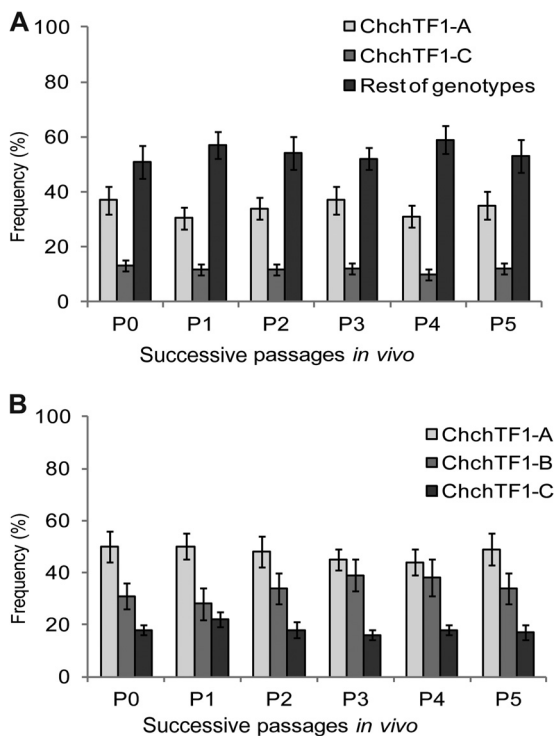

FIG 4 Relative frequencies of individual genotypes in the ChchTF1-ABC co-occluded mixture (A) and the wild-type ChchTF1 population (B) across five serial passages *in vivo* (P$_0$ to P$_5$). The error bars indicate standard errors.

sequences of these genotypes were determined and confirmed that the region of highest variability is located in these two genes. The fastest-killing genotypes, ChchTF1-B and ChchTF1-C, had an insertion in the *hoar* gene, whereas the most productive genotype, ChchTF1-A, had an insertion in the *bro-d* gene. The *bro* genes, or baculovirus repeated open reading frames (ORFs), play an important role in viral replication due to their nucleic acid binding activity and association with nucleosomes (43). Recombinant genotypes with these genes deleted or interchanged are currently being constructed with the aim of determining their roles in the observed phenotypes of ChchSNPV genotypes.

Phenotypic characterization revealed that the genotypic structure of ChchTF1-wt maximized viral pathogenicity and transmissibility, as none of the pure genotypes was more pathogenic or produced more OBs per insect than the wild-type population. ChchTF1-wt was 8.3-fold more pathogenic than the most prevalent genotype, ChchTF1-A. Single genotypes had similar levels of OB pathogenicity but differed markedly in speed of kill, as observed in other nucleopolyhedrovirus populations (6). Fast-killing genotypes tend to produce fewer OBs, probably because insects die soon after infection and have insufficient time to grow as large as larvae infected by slower-killing genotypes. However, this trade-off is not always observed in single genotypes (42). In the cases of ChchTF1-F, ChchTF1-G, and ChchTF1-H, the genotypes were as fast killing as the wild type but yielded significantly fewer OBs from each infected larva, suggesting the existence of a genetic basis for this important trait.

The fact that ChchTF1-wt OBs were more pathogenic than any of the single genotypes strongly suggested that interactions between the individual genotypes increased the pathogenicity of the wild-type population. Indeed, in OB and coocluded mixtures containing the most abundant genotypes, together with the least common ones, ChchTF1-ABCHG and ChchTF1-ABCGH, at frequencies similar to those at which they were isolated, the pathogenicity of the viral population was restored. Although constraints on the design of specific primers for rare genotypes limited our ability to generate direct evidence of the presence of rare genotypes in experimental mixtures, the qPCRs based on the viral *DNA-polymerase* gene provided indirect but consistent evidence of the presence of the rare genotypes in experimental genotype mixtures at approximately the proportions at which they were present in inocula used to initiate infections.

Similarly, studies with complete and defective genotypes of *S. frugiperda* MNPV revealed that, when they were mixed at nearly natural frequencies and coocluded into OBs, the pathogenicity of the wild-type population was restored, demonstrating cooperation between genotypes (21, 23). Interestingly, in the present study, OB mixtures and coocluded mixtures containing only the most abundant genotypes (ChchTF1-ABC) resulted in an increase in the pathogenicity of OBs with respect to ChchTF1-wt by 2-fold. Synergistic effects between genotypes have also been observed in other wild-type nucleopolyhedrovirus populations (12, 15).

In terms of virulence, determined as speed of kill, mixtures that included two of the fastest-killing genotypes (ChchTF1-B and ChchTF1-C) were significantly more virulent than the wild-type population, which was associated with decreased OB production. However, genotypes ChchTF1-A, ChchTF1-G, and ChchTF1-H, despite being the least virulent genotypes, had no significant influence on the speed of kill of mixtures, including those that contained ChchTF1-B and ChchTF1-C. When more genotypes were

added to the ChchTF1-ABC mixture, OB pathogenicity was restored to that of the wild-type population; however, virulence and OB productivity were not affected, suggesting that interactions involving other minor genotypes not included in the experimental mixtures, such as ChchTF1-D, ChchTF1-E, or other genotypes that we failed to isolate, could attenuate the speed of kill of ChchTF1-wt and increase OB productivity. The capacity to infect a host (OB pathogenicity) is crucial for virus survival, as well as the production of progeny OBs for efficient transmission (44). In this kind of obligate-killing parasites, there is often a trade-off between virulence and progeny production (12, 44, 45), and ChchTF1-wt provides a further example of this balance. Even though single genotypes were more virulent, the ChchTF1-wt population seems to be genotypically structured to maximally exploit host resources, as reflected in investment in OB production, thus increasing the likelihood of transmission under natural conditions.

One drawback for the commercialization of these viruses as biological insecticides is their low speed of kill compared to most synthetic insecticides. However, this trait can be significantly improved by manipulation of the genotype composition (46). Improvement of the speed of kill has been one of the major research objectives in the development of recombinant baculoviruses as the basis for bioinsecticidal products. Two main approaches have been employed—the expression of insecticidal toxins, enzymes, or hormones (47, 48, 49) or the deletion of genes affecting the life stage (50)—or a combination of both (51). In the present study, we demonstrated that coocclusion of certain genotypes within the same OB resulted in a significant improvement in the speed of kill. The reasons for the higher speed of kill of coocluded mixtures than of OB mixtures have not been investigated, but we hypothesize that because coocclusion results in intimate physical proximity of occlusion-derived virions, even in single-nucleocapsid NPVs, this may favor the entry of different genotypes in midgut cells at frequencies similar to those at which they were present in the inoculum, which is probably less likely to occur when genotypes are segregated among different OBs.

Notably, the coocluded genotype mixtures of ChchSNPV maintained their genotype frequencies and insecticidal characteristics over five successive passages. In contrast, in studies with genotype populations of multicapsid NPVs that had been mixed at nonnatural frequencies, genotypic mixtures rapidly converged to natural frequencies during serial passage and only maintained stable genotype frequencies once the proportions of genotypes in the natural population had been reached (52, 53).

Baculovirus-based bioinsecticides are normally used for inundative applications, in which large quantities of OBs are applied for the rapid suppression of the pest without the explicit requirement for virus transmission from progeny OBs produced by the first wave of infected larvae. Therefore, the selection of active material in insecticide development is usually focused on the isolate(s) with the most favorable pathogenicity and virulence characteristics with the aim of rapidly achieving high levels of lethal infection of the insect pest following the application of the lowest effective concentration of OBs, thus reducing the quantity of OBs and therefore the costs associated with each virus application. In this respect, the ChchTF1-ABC coocluded mixture of genotypes presented the most suitable combination of characteristics for use as a biological insecticide, better than those of the wild-type isolate. For the first time, we demonstrated that just by cooccluding three genotypes, the insecticidal properties were improved com-

pared to the wild-type population. In conclusion, the cooccluded mixture ChchTF1-ABC was found to have insecticidal characteristics and genetic and biological stability that favor its use as the basis for a biological insecticide for control of *C. chalcites* in the Canary Islands. This genotypic mixture is now undergoing field trials and virus production studies with the aim of producing a commercially viable product in the near future.

ACKNOWLEDGMENTS

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