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Recombination of Retrotransposon and Exogenous RNA Virus Results in Nonretroviral cDNA Integration

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Retroviruses have the potential to acquire host cell–derived genetic material during reverse transcription and can integrate into the genomes of larger, more complex DNA viruses. In contrast, RNA viruses were believed not to integrate into the host's genome under any circumstances. We found that illegitimate recombination between an exogenous nonretroviral RNA virus, lymphocytic choriomeningitis virus, and the endogenous intracisternal A-type particle (IAP) retrotransposon occurred and led to reverse transcription of exogenous viral RNA. The resulting complementary DNA was integrated into the host's genome with an IAP element. Thus, RNA viruses should be closely scrutinized for any capacity to interact with endogenous retroviral elements before their approval for therapeutic use in humans.

Lymphocytic choriomeningitis virus (LCMV) is an ambisensed RNA virus belonging to the Arenaviridae family with no known reverse transcriptase (RT) activity. Nevertheless, DNA complementary to the small genomic RNA of LCMV, which encodes for the viral glycoprotein (GP) and nucleoprotein (NP), is found in vitro and in vivo after infection (*1*).

Intracisternal A-type particle (IAP) elements, retrotransposons that belong to the retroviral family, are found in ~1000 copies per haploid genome of the mouse (*Mus musculus*). Only a fraction of these is assumed to be actively transposing (*2*). Their retrotransposition has frequently been associated with oncogenesis or aberrant cytokine expression (*3*–*7*) reviewed in (*8*). IAP expression has been associated with tumor control (*9*) or major histocompatability complex class I down-regulation (*10*). Transcription of IAP genes, and probably the transposition of IAP, occurs during spermatogenesis and in the thymus (*11*, *12*). To date, the biological role of IAP elements and other endogenous retroviruses remains unclear.

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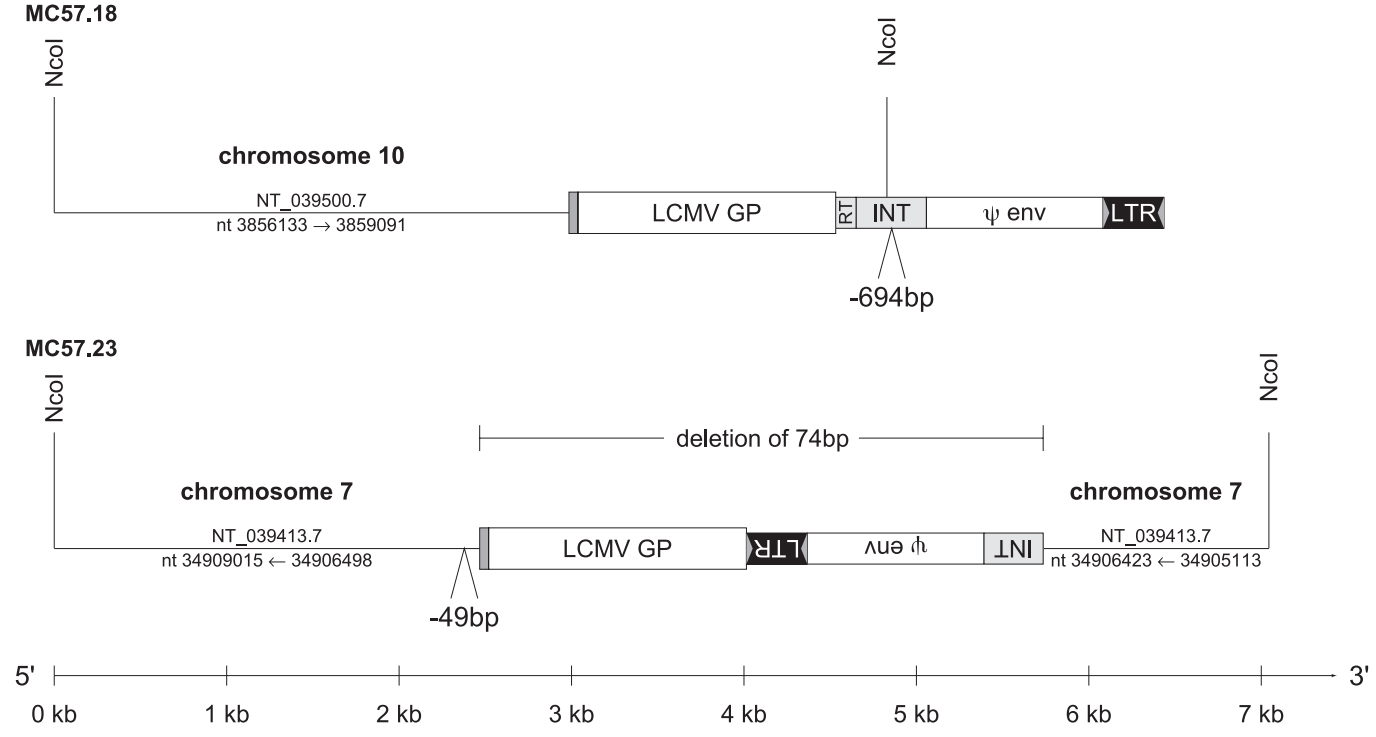


Fig. 1. DNA complementary to LCMV-GP integrated into the genome in conjunction with sequences derived from a retrotransposon. Schematic maps of cGP and flanking sequences in clone MC57.18 and MC57.23 derived from LCMV infected MC57G cells. Genes, open or gray boxes; IAP LTRs, black boxes with the inverted repeats indicated as gray triangles. Positions of deletions and restriction sites are indicated.

Here, we show that endogenous IAP elements promoted reverse transcription and integration of LCMV RNA after infection. These findings impinge on the design of RNA vectors and indicate alternative mechanisms for the coevolution between host, endogenous retroviral elements, and exogenous viruses.

Formation of DNA complementary to the LCMV RNA genome (cLCMV) after infection of MC57G cells can be detected in about 1 in 10^3 to 10^4 cells (*1*). To analyze the events leading up to cLCMV formation, we serially subcloned LCMV-infected MC57G cells to derive two stable cell lines (MC57.18 and MC57.23) that contained genetic material complementary to LCMV GP (cGP) (Fig. 1) (*13*). It is important to note that LCMV could not be detected in the supernatants of these cell cultures after the subcloning procedure (*14*).

Using a combination of long-range and inverse polymerase chain reaction (PCR) (*13*), we isolated the LCMV-GP–derived genetic material, including the flanking sequences from both clones (Fig. 1). In both cell lines, the virus-derived genetic material consisted of a full-length cGP gene that was joined at the 3′ end to sequences derived from murine IAP retrotransposon (Fig. 1). In both cases, homology with IAP ended within the 3′ region of the IAP polymerase/integrase gene, albeit at different locations and in opposite orientations. The 5′ cGP-flanking sequences of MC57.18 shared 99% homology with the C57BL/6 mouse chromosome 10 (NT_039500.7, nucleotides 3856133 to 3859091). The 5′ and 3′ cGP flanking sequences of MC57.23 were 99% homologous to chromosome 7 from C57BL/6 mice (NT_039413.7, nucleotides 34906498 to 34909015 and 34905113 to 34906423, respectively), with an internal deletion of 49 base pairs near the integration site.

Analysis of the homologies between the cGP sequences and the small genomic RNA of LCMV revealed that both the 3′ and 5′ homology breakpoints were almost at the same position [± 1 nucleotide (nt)] (fig. S1, A and B). At the 5′ end, homology with LCMV always ended in the 5′ untranslated region. Both 3′ homology breakpoints were located within the stop codon of the LCMV-GP open reading frame. No target site duplication was found at the site of integration in MC57.23, suggesting that integration of the cLCMV/IAP sequences was not catalyzed by a classical retroviral integrase activity. In addition, the multiple deletions shown in Fig. 1 indicate that integration of the cLCMV/IAP recombination product might have occurred at sites of DNA damage [supporting online material (SOM) text 1].

From these results, we hypothesized that IAP elements were catalyzing the reverse transcription and subsequent integration of the cGP genes. To obtain more formal proof, we transfected a series of plasmids encoding for functional IAP retrotransposons (213P12, 440N1, 92L23, and 262J2) (*15*) into LCMV-infected cell lines from four different species (human, green monkey, dog, and Chinese hamster) that do not display cGP formation (*1*) after LCMV infection (Table 1). As a control, we transfected a plasmid encoding enhanced green fluorescence protein (EGFP) or we left the cells untreated. Three days later, we isolated genomic DNA and tested for the presence of cGP by nested PCR (Table 1).

Untransfected or LCMV-infected cells transfected with the control EGFP plasmid did not make cGP [0 out of 34 independent transfection experiments (0/34)]. Transfection of non-infected cells with the panel of IAP elements also failed to produce cGP sequences (0/47). In contrast, transfection of LCMV-infected cells with IAP-expressing plasmids resulted in the formation of cGP sequences with a frequency of 70.2% (33/47). Some IAP variants (e.g., 262J21 and 440N1) mediated cGP formation more efficiently (≥80%) than others (e.g., 92L23, with 40% efficiency), confirming that expression of functional IAP elements is required for the formation of LCMV cDNA.

IAP elements lacking (440N1ΔRT) or having a nonfunctional (440N1LinkerStopRT) *pol* gene failed to mediate cGP formation in LCMV-infected Vero cells (Table 2). We also found that in the absence of gag, cGP formation did not occur (0/2).

Table 1. Correlation between IAP expression and LCMV cDNA formation. Human (HeLa), green monkey (Vero), canine (MDCK), and Chinese hamster (CHO) cells were infected with LCMV at a multiplicity of infection of 0.001 (+) or left untreated (–). One day later, plasmids expressing the indicated functional IAP element (*15*) or a control plasmid expressing EGFP were transfected. The number of cultures displaying cGP formation per total number of cultures tested is indicated.

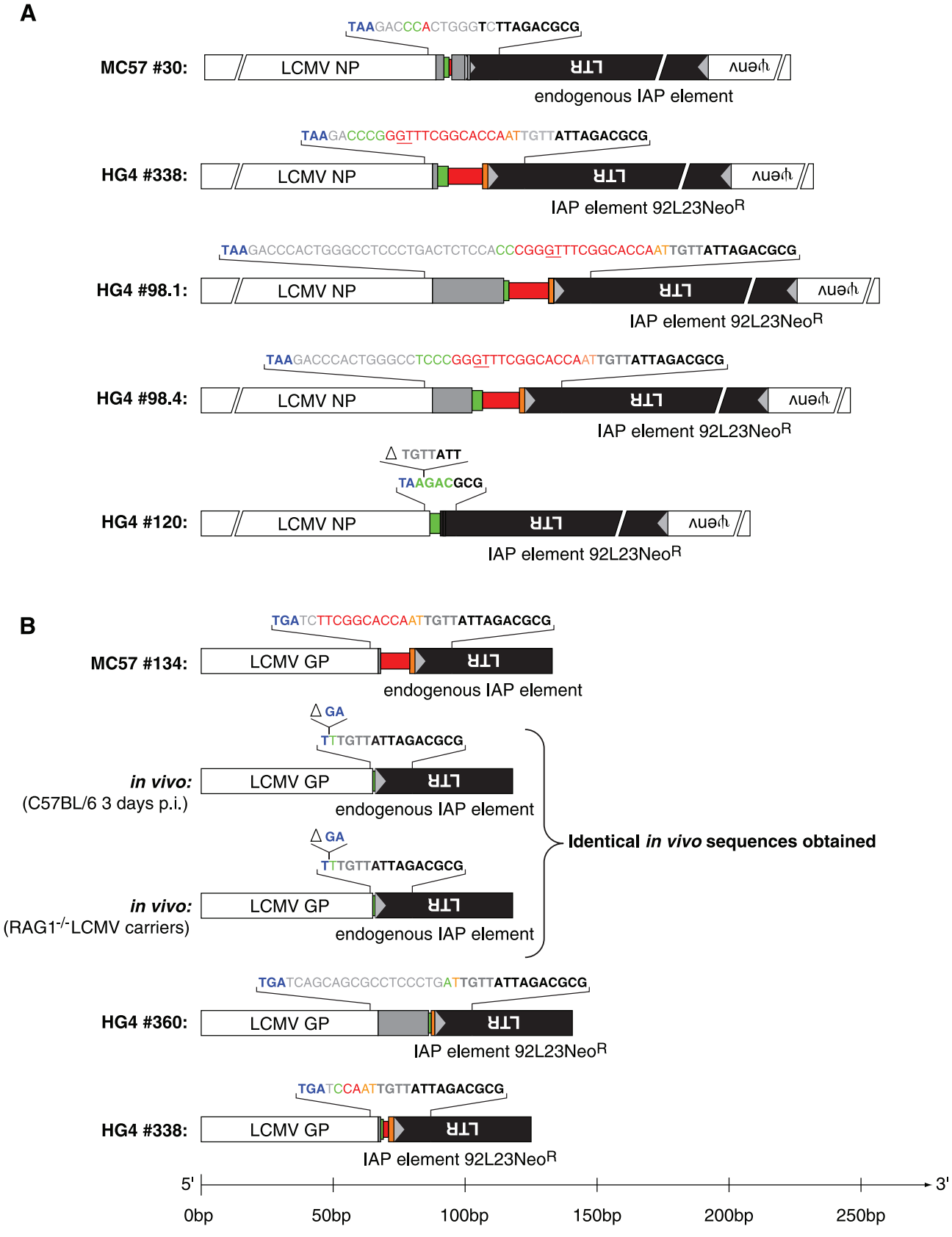
Virus/cell line	Plasmids containing functional wild-type IAP elements								Negative controls			
	213P12		440N1		92L23		262J21		pEGFP-N3		Mock	
LCMV	–	+	–	+	–	+	–	+	–	+	–	+
HeLa	0/3	3/3	0/3	3/3	0/3	2/3	0/5	4/5	0/5	0/5	0/5	0/5
Vero	0/2	0/2	0/2	1/2	0/2	0/2	0/5	4/5	0/5	0/5	0/5	0/5
MDCK	0/3	2/3	0/3	2/3	0/3	0/3	0/5	5/5	0/5	0/5	0/5	0/5
CHO	0/2	2/2	0/2	2/2	0/2	2/2	0/2	1/2	0/2	0/2	0/2	0/2
Total	0/10	7/10	0/10	8/10	0/10	4/10	0/17	14/17	0/17	0/17	0/17	0/17
% cGP+	0%	70%	0%	80%	0%	40%	0%	82%	0%	0%	0%	0%

Table 2. IAP-derived RT activity is required for LCMV GP cDNA formation. Vero cells were infected with LCMV at a multiplicity of infection of 0.001 (+) or left untreated (–). One day later, plasmids containing a functional IAP element (440N1), RT-deficient IAP elements (440N1ΔRT and LinkerStopRT), or the HIV-1 Gag-Pol plasmid pCMVΔR8 were transfected. Mock transfection was performed as an additional negative control. The number of cultures displaying cGP formation per total number of cultures tested is indicated.

Virus/cell line	Functional		No functional IAP RT				HIV-1 *Gag-Pol*		Control	
	440N1		440N1ΔRT		LinkerStopRT		HIV-1 pCMVΔR8		Mock	
LCMV	–	+	–	+	–	+	–	+	–	+
Vero	0/10	11/14	0/1	0/16	0/4	0/8	0/4	1/18	0/8	0/12
% cGP+	0%	78.6%	0%	0%	0%	0%	0%	5.6%	0%	0%

Fig. 2. cNP/IAP and cGP/IAP recombinations detected in vitro and in vivo in mice after LCMV infection. Recombination products for cNP/IAP (**A**) and cGP/IAP (**B**) amplified from genomic DNA of the indicated source and sample number. Genes are shown as labeled open boxes, gray boxes indicate insertion sequences derived from the intergenic region of the small genomic RNA of LCMV, black boxes indicate IAP LTR-derived sequences, and a gray triangle indicates the inverted repeats of the IAP LTR. Gray normal-faced letters represent nucleotides derived from the LCMV intergenic region, and bold-faced ones represent the direct repeat of the IAP LTR. Red boxes and letters indicate sequences derived from $tRNA^{Phe}$. The underlined nucleotides GT are only found in the $tRNA^{Phe}$ and not in the IAP primer-binding site. Blue bold letters indicate the NP and GP stop codons. Orange letters and boxes indicate the nucleotides bridging between the IAP LTR and the primer-binding site (fig. S2). Green letters and boxes indicate nucleotides at the homology break points that cannot unambiguously be assigned to either $tRNA^{Phe}$/IAP LTR or LCMV small RNA. The center of the maps showing the recombination site is proportional to the depicted ruler, but large portions of the LCMV NP, IAP LTR, and IAP ψenv genes were omitted because of limited space and are indicated by interrupted gene symbols (A).

When we compared the ability of gag/pol proteins derived from IAP to mediate cGP formation with that of gag/pol proteins derived from another retrovirus (HIV-1, pCMVΔR8) (*16*), we found that the IAP element 440N1 was more efficient (11 out of 14 = 78.6%) than HIV-1 (1 out of 18 = 5.6%) (Table 2) in catalyzing cGP formation. Moreover, the guinea pig cell line CCL158 fails to form cLCMV, despite high levels of RT activity (*1*). Thus, the efficiency of the endogenous retrovirus derived from the natural host in mediating cLCMV formation probably reflects their evolutionary history.



To further investigate the detailed molecular events giving rise to LCMV/IAP hybrid DNA formation, we designed nested PCR approaches that detect recombined cLCMV/IAP hybrid sequences (*13*). We were able to amplify both cNP/IAP and cGP/IAP recombination events directly from infected murine MC57G cells (Fig. 2). We also detected cGP/IAP recombination events in vivo in splenocytes of an acutely infected C57BL/6 mouse and in peripheral blood mononuclear cells of RAG1$^{-/-}$ mice persistently infected with LCMV (Fig. 2B), demonstrating that recombination between IAP retrotransposons and LCMV was not an artifact of immortalized cells but also occurred spontaneously in vivo.

The abundance of IAP elements in the murine genome (*2*) makes a detailed interpretation of sequence data difficult, as the exact sequence of the IAP element involved in LCMV hybrid formation is not known. We generated a recombinant HeLa cell line (HG4) in which all the IAP elements were derived from a single molecular clone 92L23Neo^{R} (*15*) and that reliably formed cLCMV upon infection.

Three days after LCMV infection of HG4 cells we amplified several different cNP/IAP and cGP/IAP recombination events involving the 92L23Neo^{R} IAP element (Fig. 2). All amplified recombination products were sequenced and analyzed for homologies to 92L23Neo^{R} and the LCMV genome. In contrast to the sequences obtained from the subcloned infected MC57G cell lines MC57.18 and MC57.23, where IAP and LCMV sequences were directly joined to each other (Fig. 1), we observed insertions of additional nucleotides at the site of recombination in cLCMV/IAP hybrid DNA amplified from infected HG4 and MC57G cells (Fig. 2). These insertions, consisting of up to 14 nts displayed homology to the 3′ end of the 76-nt-long phenylalanine tRNA (tRNA^{Phe}, GenBank accession number K02684), which has previously been shown to act as primer for the reverse transcription of IAP elements (SOM text 2) (*17*).

We showed that endogenous retroviral elements can recombine with exogenous nonretroviral RNA viruses presumably by copy choice during reverse transcription (SOM text 2) to yield cDNA complementary to both the endogenous retrovirus and the exogenous RNA virus. Such recombinant cDNA has the potential to integrate into the genome of the infected cell, and because IAP elements have been shown to transpose most efficiently in the thymus and in the LCMV-sanctuaries (*18*) of the testicles (*11*, *12*), such recombinations theoretically could lead to germline transmission of viral genes from RNA viruses. Therefore, nonretroviral RNA viruses may have contributed more substantially and directly to the evolution of mammalian genomes than has been assumed so far (SOM text 3). However, extensive database analysis of the murine genome, which was determined with DNA from LCMV-free mice, did not yield substantial homologies to the LCMV-genome. In humans, there is some evidence to suggest that at least one subgroup of the human endogenous retrovirus-K family may be active (*19*, *20*). Thus, the potential risk of somatic integration into host cells during human gene therapy using RNA virus vectors should be experimentally assessed by nested PCR.

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Supporting Online Material

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Molecular Mechanisms of HipA-Mediated Multidrug Tolerance and Its Neutralization by HipB

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Bacterial multidrug tolerance is largely responsible for the inability of antibiotics to eradicate infections and is caused by a small population of dormant bacteria called persisters. HipA is a critical *Escherichia coli* persistence factor that is normally neutralized by HipB, a transcription repressor, which also regulates *hipBA* expression. Here, we report multiple structures of HipA and a HipA-HipB-DNA complex. HipA has a eukaryotic serine/threonine kinase–like fold and can phosphorylate the translation factor EF-Tu, suggesting a persistence mechanism via cell stasis. The HipA-HipB-DNA structure reveals the HipB-operator binding mechanism, ~70° DNA bending, and unexpected HipA-DNA contacts. Dimeric HipB interacts with two HipA molecules to inhibit its kinase activity through sequestration and conformational inactivation. Combined, these studies suggest mechanisms for HipA-mediated persistence and its neutralization by HipB.

Bacteria that are resistant or tolerant to antibiotics are an increasing threat to human health. Indeed, ~60% of infections in the developed world are caused by biofilms, which exhibit multidrug tolerance (MDT) (*1*, *2*). MDT is caused by the presence of dormant bacterial cells called persisters, which account for only 10^{-6} to 10^{-4} cells in a growing population, making MDT difficult to study (*3*–*5*). Persisters are not mutants but phenotypic variants of wild-type cells that evade killing by somehow adopting a transient dormant state (*6*, *7*). Dormancy provides protection because bactericidal antibiotics kill by corrupting their active targets into producing toxic byproducts. These protected persisters can then switch back to the growth phase after the removal of antibiotics, allowing the bacterial population to survive. The first high-persistence allele, *hipA7*

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