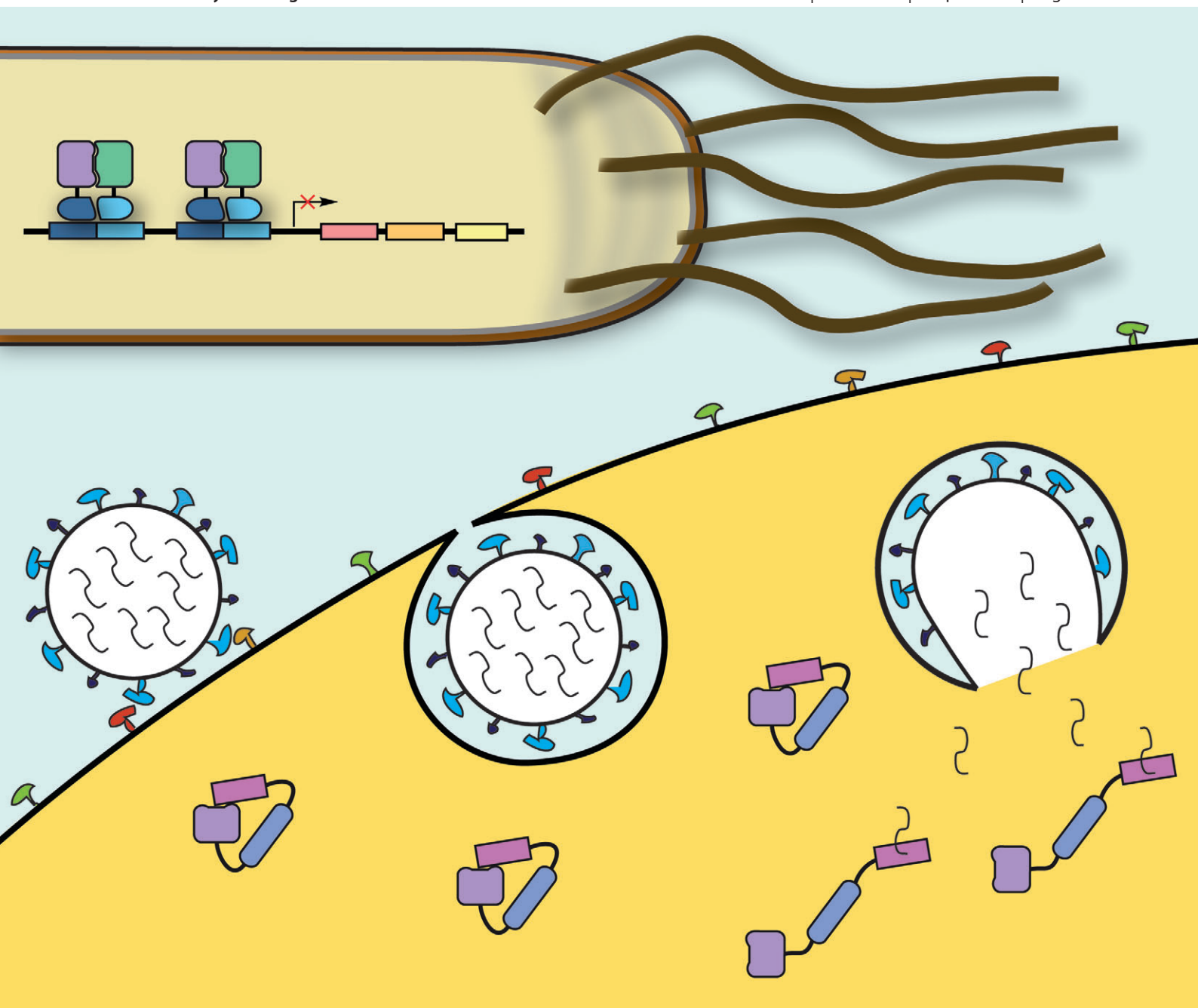


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**COMMUNICATION**

Ali Tavassoli *et al.*

Deciphering interactions used by the influenza virus NS1 protein to silence the host antiviral sensor protein RIG-1 using a bacterial reverse two-hybrid system

## Deciphering interactions used by the influenza virus NS1 protein to silence the host antiviral sensor protein RIG-I using a bacterial reverse two-hybrid system†

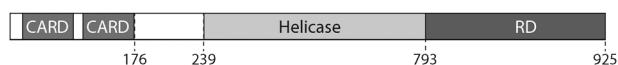
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The majority of biological processes are controlled and regulated by an intricate network of thousands of interacting proteins. Identifying and understanding the key components of these protein networks, especially those that play a critical role in disease, is a challenge that promises to dramatically alter our current approach to healthcare. To facilitate this process, we have developed a method for the rapid construction of a chromosomally integrated, bacterial reverse two-hybrid system (RTHS) that enables the identification of interacting protein partners. Chromosomal integration of the RTHS enables stable protein expression, free of plasmid copy-number effects, as well as eliminating false positives arising from plasmid ejection. We have utilized this approach to identify the interactions used by the influenza virus NS1 protein to silence the host's antiviral defences.

Mammalian cells contain several proteins dedicated to detecting and responding to viral infection. The RNA helicase, retinoic acid inducible gene-I (RIG-I), acts as the sensor for influenza and other negative strand RNA viruses. Upon detection of viral RNA, the innate immune response is characterized by the rapid production of a range of cytokines, most prominently type I interferon (IFN- $\alpha/\beta$ ),<sup>1</sup> which act to reduce viral replication and spread.<sup>2</sup> RIG-I is a 106 kDa protein that consists of 3 functional domains (Fig. 1).<sup>3–5</sup> The N-terminal region contains two caspase activation and recruitment domains (CARD) that interact with the downstream effectors of RIG-I and activate interferon production. The first CARD was recently shown to bind the E3 ubiquitin ligase, tripartite motif protein 25 (TRIM25), facilitating the ubiquitination of the second CARD at Lys172 (an essential modification for



**Fig. 1** The domains of RIG-I. The N-terminus of RIG-I contains two CARDs (residues 1–176), followed by a linker region, and helicase (residues 239–793). The C-terminus of the protein (residues 793–925) contains the regulatory domain (RD).

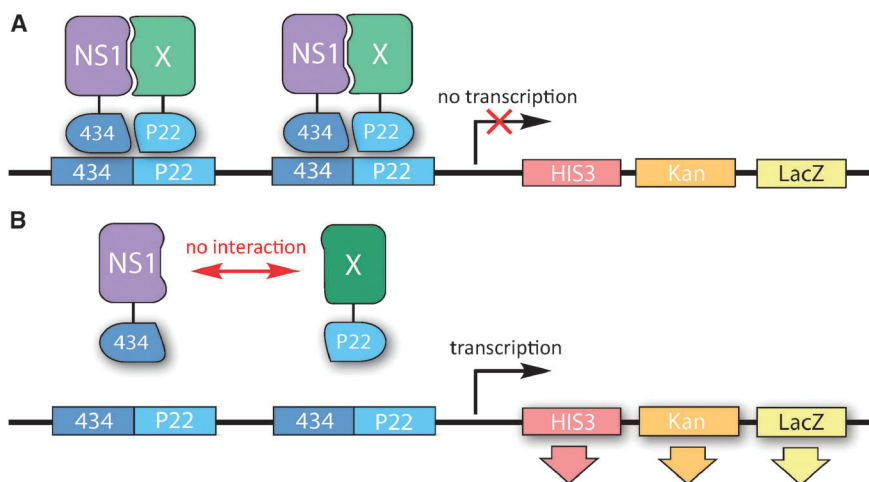
IFN induction).<sup>6</sup> The central domain of RIG-I is the DEXH box RNA helicase/adenosine triphosphatase domain (helicase), and is thought to be responsible for unwinding the viral genome.<sup>7</sup> The C-terminal regulatory domain (RD) recognises and binds viral RNA. In the absence of viral RNA, RD interacts with CARD to keep RIG-I in a closed conformation, preventing signalling. Upon detection of viral RNA by RD, RIG-I undergoes a change in conformation to expose CARD,<sup>3</sup> activating the cascade of downstream effectors that initiate the production of type I interferon.<sup>8,9</sup>

Viruses have evolved multiple mechanisms to inactivate the viral sensors of the host.<sup>10</sup> In the case of influenza virus, the 26 kDa non-structural protein 1 (NS1) is widely regarded as a factor that antagonizes the host's immune responses,<sup>11,12</sup> NS1 deficient viruses are highly attenuated and only replicate efficiently in IFN-deficient systems.<sup>13</sup> NS1 function relies on its ability to participate in a multitude of protein–protein and protein–RNA interactions, and although RIG-I is known to be targeted by NS1,<sup>13</sup> the exact nature of their interaction is unclear. NS1 has been shown to co-precipitate with RIG-I indicating a direct interaction between the two proteins, but it has been suggested that the interaction may be mediated by a third factor.<sup>1</sup> The recent identification of TRIM25 as a binding partner of NS1 has resulted in an alternative hypothesis for the mechanism of NS1 inactivation of RIG-I, where the NS1–TRIM25 interaction inhibits TRIM25 multimerization, therefore preventing the ubiquitination of RIG-I CARD, suppressing RIG-I signal transduction and ultimately IFN production.<sup>6</sup>

To enable the facile probing of these (and other) protein–protein interactions, we constructed a two-plasmid system (an integration vector and a helper plasmid) that allows the rapid assembly of a chromosomally integrated, bacterial RTHS (Fig. 2). The RTHS has been previously reported,<sup>14,15</sup> and is based on the bacteriophage regulatory system, linking the disruption of the interaction between the two targeted proteins, expressed as hybrid fusions of a chimeric repressor genes, expressed as hybrid fusions of a chimeric repressor genes. The reporter construct (present on the chromosome of the host strain SNS126)<sup>14</sup> includes the chimeric 434/P22 operators followed by HIS3 (imidazole glycerol phosphate dehydratase) and Kan<sup>R</sup> (aminoglycoside 3'-phosphotransferase for kanamycin resistance) genes. SNS126 lacks the native

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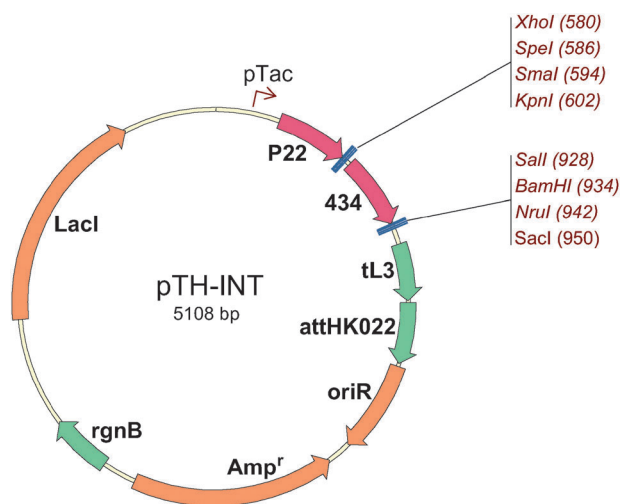
**Fig. 2** Monitoring protein–protein interactions with a bacterial reverse two-hybrid system. (A) If the targeted proteins interact (NS1, and X = RIG-I, CARD, helicase, RD or TRIM25) a functional repressor will be reconstituted that prevents the transcription of the reporter genes downstream. This will lead to cell death on selective media. (B) If the targeted proteins do not interact, the 434.P22 repressor is not functional, allowing the transcription of the reporter genes. This will lead to growth on selective media.

HISB gene and requires expression of the yeast HIS3 gene for histidine biosynthesis. The stringency of both reporter genes is chemically tuneable (with 3-amino-1,2,4-triazole and kanamycin), and both gene products are required for host survival on selective media. The third reporter gene, LacZ encodes  $\beta$ -galactosidase and is used to quantify the targeted protein–protein interaction through *o*-nitrophenyl- $\beta$ -galactoside (ONPG) assays.

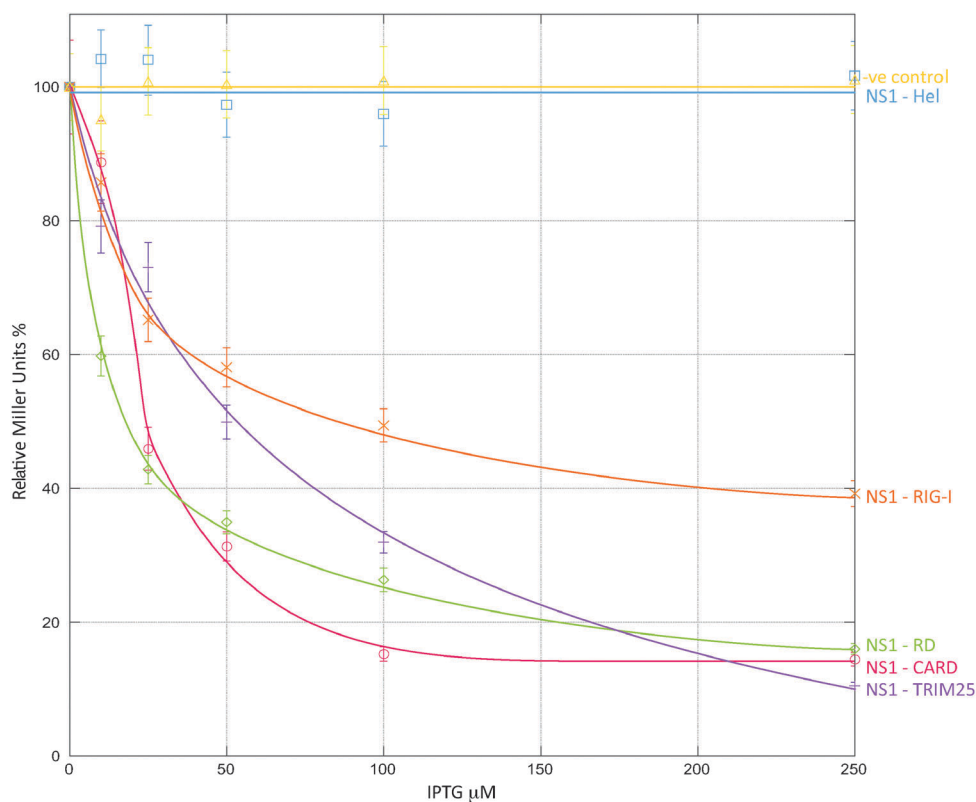
The integration vector encodes the bacteriophage 434 repressor DNA binding domain, and a chimeric P22 variant of 434 (from pTHCP14)<sup>14</sup> each followed by a polylinker for insertion of the targeted proteins (Fig. 3). The plasmid also contains a conditional  $\gamma$  replication origin of R6K, and an HK022 phage attachment site (both from pAH68).<sup>16</sup> The R6K origin requires the *trans*-acting II protein (encoded by *pir*) for

plasmid replication, enabling its over-expression during cloning in *pir*<sup>+</sup> strains of DH5 $\alpha$  *E. coli* (DH5 $\alpha$ -*pir*). As the plasmid is not replicated in SNS126 (non-*pir*), integrants are distinguishable from transformants. Integration of pTH-INT into the HK022 attachment site of SNS126 occurs in the presence of the HK022 integrase, whose expression from the helper plasmid (pAH69T) is controlled by *cI857*,<sup>17</sup> and is induced at elevated temperatures. The helper plasmid is a derivative of pAH69,<sup>16</sup> with BLA (ampicillin resistance) replaced with TETA (tetracycline resistance) to enable selection for integrants containing pTH-INT.

We used this system to determine the protein–protein interactions that are utilized by Influenza A NS1 to silence the host's viral sensors. We reasoned that the absence of direct homologues of the human viral sensor pathway proteins in bacteria would minimise the possibility of native proteins inadvertently mediating the interaction of NS1 with RIG-I/TRIM25 and leading to false-positive data. As all the target proteins are routinely over-expressed in *E. coli*, we were confident that they would express and fold correctly in our bacterial RTHS, reducing the likelihood of false-negatives. The gene encoding Influenza A NS1 was cloned into the pTH-INT plasmid as an N-terminal fusion with 434, while RIG-I or TRIM25 was cloned as an N-terminal fusion with P22. The resulting plasmids were integrated into the chromosome of the RTHS strain (SNS126) by phage-specific recombination using the HK022 phage integrase, as previously described.<sup>16</sup> The resulting RTHS strains (NS1–RIG-I and NS1–TRIM25) and a negative control strain expressing only 434 and P22 were used to determine and quantify the extent of each protein–protein interaction. We used a previously built RTHS as the positive control.<sup>18</sup> Upon addition of IPTG, the targeted proteins are expressed as fusions with 434 or P22, from the chromosomally integrated pTH-INT cassette. The interaction of the IPTG-induced proteins reconstitutes a functional repressor that prevents transcription of reporter genes downstream (Fig. 2A). If the target proteins do not interact, the host cells will express the reporter genes regardless



**Fig. 3** The RTHS plasmid pTH-INT. The plasmid includes two multiple cloning sites used for the cloning of the targeted proteins into the RTHS. Protein expression is controlled *via* a pTac promoter, with a copy of LacI<sup>q</sup> included to enable stringent IPTG induction of the RTHS proteins. The plasmid also contains an HK022 phage attachment site (attHK022), and a P6K replication origin (oriR).



**Fig. 4** ONPG assay. The targeted protein–protein interactions of NS1 were quantified using ONPG assays. IPTG increases cellular levels of the targeted proteins (expressed as fusions with 434 or P22 repressor domains, see Fig. 2), leading to reduced expression and eventual shutdown of the *LacZ* reporter gene.  $\beta$ -galactosidase levels (*LacZ* gene product) are monitored by ONPG assays. The data indicate an interaction between NS1 and RD, TRIM25 and CARD, and no interaction between NS1 and helicase.

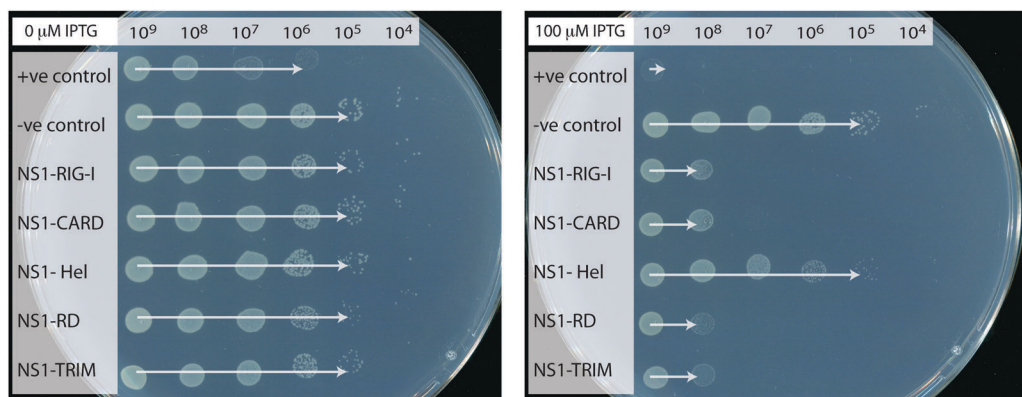
of IPTG levels (Fig. 2B). We quantified the protein–protein interaction of each NS1 RTHS using *o*-nitrophenyl- $\beta$ -galactoside (ONPG) assays. The data (Fig. 4) indicated an interaction between NS1 and TRIM25 (reduction in *LacZ* gene product as IPTG levels increased), in agreement with the recently reported co-immunoprecipitation studies.<sup>6</sup> We also observed a direct interaction between NS1 and RIG-I, which the data suggested is weaker than that between NS1 and TRIM25. The lower affinity may be an artefact of the RTHS and a consequence of the larger size of RIG-I (compared to TRIM25) preventing the optimal interaction of the 434 and P22 repressor domains. We identified the domains of RIG-I that are bound by NS1 by constructing RTHS for the NS1–CARD, NS1–helicase and NS1–RD. The ONPG assays of the resulting RTHS strains (Fig. 4) identified CARD and RD as the domains of RIG-I that are targeted by NS1.

We visualized the targeted protein–protein interactions by drop spotting ten-fold serial dilutions of the above NS1 RTHS strains (with positive and negative controls) onto selective media containing 3-amino-1,2,4-triazole (2.5 mM) and kanamycin (25  $\mu\text{g mL}^{-1}$ ) in the absence or presence of IPTG (100  $\mu\text{M}$ ). The drop spotting data (Fig. 5) confirmed the interaction between NS1–RIG-I, NS1–CARD, NS1–RD and NS1–TRIM25, correlating with the above ONPG assays. To eliminate the possibility that the observed growth inhibition is due to the toxicity of the expressed proteins, the RTHS strains were drop spotted onto rich media (LB agar) with 100  $\mu\text{M}$  IPTG. All strains grew normally under these conditions

(ESI<sup>†</sup>); the observed growth inhibition on selective media can therefore be attributed to the targeted protein–protein interactions.

In summary, we have developed a method for the rapid construction of bacterial RTHS and have used this approach to identify the direct interaction of NS1 with RIG-I, by binding to its CARD and RD domains. These observations (when combined with currently available data) suggest that NS1 binds to RIG-I in its closed conformation, in which the RD and CARD domains are known to be in close proximity. Such an interaction would stabilize the closed confirmation of RIG-I, masking its viral genome recognition site and preventing the conformational change to the active form. The ability of NS1 to bind CARD also suggests that the viral protein may additionally bind the active form of RIG-I, directly interfering with its subsequent downstream signalling. Our data also confirm the interaction of NS1 with TRIM25 that was previously reported.<sup>6</sup> These observations taken together lead us to suggest that the postulated mechanisms for NS1 inhibition of RIG-I are not mutually exclusive, and that NS1 targets the antiviral RIG-I signaling pathway at multiple points through its interaction with TRIM25, CARD and RD, demonstrating the robust manner in which Influenza A NS1 silences the host's antiviral sensors. The reported two-plasmid system may also be used to probe other protein–protein interactions, however (to avoid false positives) the expression of functional target proteins in bacteria should be confirmed prior to constructing the RTHS. We are currently using the above





**Fig. 5** Drop spotting of the NS1 RTHS strains. Serial dilutions ( $2.5 \mu\text{L}$  of  $\sim 10^9$  cells  $\text{mL}^{-1}$ ) of the NS1 RTHS strains (and controls) were drop spotted onto selective media containing 0 or 100  $\mu\text{M}$  IPTG (plate on left and right respectively). In the absence of IPTG, there is no expression of the targeted fusion proteins; all strains therefore grow normally (plate on the left). Upon addition of IPTG (plate on right), the functional 434.P22 repressor will be reconstituted in those RTHS that contain interacting proteins (Fig. 2A), leading to cell death. The strains containing non-interacting partners will continue to grow normally. The above data show that influenza A NS1 interacts with RIG-I, CARD, RD, and TRIM25, as demonstrated by cell death upon IPTG induction of the fusion proteins. Positive control is the P6-UEV RTHS<sup>18</sup> and negative control is the 434 and P22 repressor domains alone.

NS1 RTHS in high-throughput screens to identify compounds that disrupt these critical protein–protein interactions, for use as chemical tools to allow the role of each of these interactions to be deciphered in cells.

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