INTRODUCTION

Interaction of pathogenic bacteria with host cells is often facilitated by dedicated protein secretion systems that have evolved to deploy their respective substrate proteins as pathogenicity determinants. A common theme in this respect in Gram-negative bacteria is the direct injection of effector proteins into target cells, which is mediated by type III, type IV, or type VI secretion systems (Galán & Waksman, 2018). In addition, some type IV and type VI secretion systems are used to transport effector proteins into other bacterial cells. Type IV secretion systems (T4SS) are known as macromolecule transporters with a wide range of transported substrates, from small monomeric effector proteins to nucleoprotein complexes of considerable size. As an adaptation to different substrates, functions and host cell types, the corresponding transporters also vary considerably in their composition and the mechanistic details of substrate transport (Li et al., 2019). Transport capabilities range from only one or a few to several hundred different effector proteins. The Cag T4SS of the human gastric pathogen Helicobacter pylori is at an extreme position within this range, as it represents an expanded T4SS containing several non-canonical components in addition to the core subunits (Costa et al., 2020), but seems to transport only one effector protein, the cytotoxin-associated antigen CagA, into gastric target cells (Cover et al., 2020).

Translocation of the CagA protein to gastric epithelial cells is considered a hallmark of H. pylori pathogenesis, and due to the...
RESULTS

2.1 Evaluation of a NanoLuc HiBiT fusion as CagA type IV secretion reporter

Several epitope tags or protein domains with different amino acid sequences have been demonstrated to be co-translocated to target cells, when fused to the N-terminus of CagA (Hohlfeld et al., 2006; Murata-Kamiya et al., 2010; Schindele et al., 2016). To examine the possibility of utilizing a split-luciferase system for monitoring translocation, we generated an \textit{H. pylori} strain producing a CagA variant with an N-terminal NanoLuc HiBiT tag by integrating the HiBiT sequence without a resistance marker in the \textit{cagA} locus (Figure 1a). Immunoblots of bacterial lysates that were probed with recombinant LgBiT protein confirmed that the HiBiT-CagA fusion can be easily detected via its HiBiT tag (Figure 1b). To evaluate translocation of HiBiT-CagA into target cells, we first determined HiBiT-CagA tyrosine phosphorylation upon co-incubation with AGS cells. Immunoblot analysis showed that HiBiT-CagA was indeed phosphorylated, albeit at a lower efficiency in comparison to wild-type CagA, whereas no phosphorylation was detected with a HiBiT-CagA-producing, but secretion-deficient strain (\textit{ΔcagF}; Figure 1c). In contrast, tyrosine phosphorylation of a TEM-1-CagA fusion protein was not visible under these conditions, probably due to lower protein levels, as shown previously (Schindele et al., 2016). Microscopic analysis of infected AGS cells confirmed that translocated HiBiT-CagA was also capable of eliciting the hummingbird phenotype, which is dependent on phosphorylated CagA, to a similar extent as P12 wild-type (Figure 1d). In contrast, the reporter strain producing a TEM-1-CagA fusion did not show clear signs of hummingbird cells under these conditions. To be able to measure HiBiT-CagA translocation independently of its tyrosine phosphorylation, we stably transfected AGS cells with an expression plasmid encoding the LgBiT sequence coupled to a self-labeling HaloTag (Los et al., 2008). Single transfected cell clones were selected by monitoring LgBiT production via a fluorescent HaloTag ligand, and expanded to obtain AGS (LgBiT) cells (Figure S1a). When we used these cells for standard infection experiments with \textit{H. pylori} P12 [HiBiT-CagA], and replaced the co-culture supernatant with a solution containing the NanoLuc substrate furimazine, we observed a strong luminescence, which was not seen with uninfected cells, or after co-incubation with AGS cells (Figure S1a). The luminescence signal was maximal within the first 10 min after adding the substrate, and then slowly decreased (Figure S1b). When we infected AGS (LgBiT) cells with P12 [HiBiT-CagA] at different multiplicities of infection (MOI), luminescence signals increased with bacterial numbers in a linear way over a wide range of bacteria-to-cell ratios, with no evidence of substrate or LgBiT limitation up to MOI 25 (Figure 1f). To assess the sensitivity of the HiBiT-CagA translocation assay, we determined the luminescence values at very low infection doses, and could detect signals above background down to an MOI of 0.125 (Figure 1g). These results show that the HiBiT tag is a useful reporter for detecting CagA translocation at high sensitivity, and with minimal disturbance of downstream cellular processes.
is produced at rather high levels; in fact, CagA represents one of the most abundant H. pylori proteins under standard culture conditions (Jungblut et al., 2000). However, translocation competition experiments using strains that co-produce tagged and untagged versions of CagA, and estimation of translocation by quantitative immunoblot analysis, suggested that only a minor fraction of the bacterial CagA pool gets translocated into cultured target cells (Jiménez-Soto & Haas, 2016; Schindele et al., 2016). To obtain a more quantitative view on CagA translocation rates, we first quantified translocated HiBiT-CagA after co-incubation with AGS [LgBiT] cells, as described.
above, and subsequently estimated the total HiBiT-CagA amount present in the infected cells and adherent bacteria. This was achieved by lysis of target cells together with the attached bacteria, and measuring luminescence after adding fresh substrate and recombinant LgBiT to the lysis solution (see Experimental Procedures). Using this procedure after 2.5 hr of co-incubation, we obtained high luminescence values that were designated as total HiBiT-CagA (Figure S2).

Taking into account that the amount of LgBiT intrinsically produced by the AGS cells was not limiting for measuring translocation at MOI values up to 25 (Figure 1f), we concluded that the additional luminescence obtained after lysis is derived from HiBiT-CagA retained within the bacterial cells. Thus, the lysis protocol confirmed that H. pylori cells had translocated only a small fraction of their CagA pool during this time.

To quantitatively determine the influence of bacterial cell numbers on the translocated fraction of HiBiT-CagA, we co-incubated AGS [LgBiT] cells with P12 [HiBiT-CagA] at different MOIs, and determined translocated and total HiBiT-CagA values with this procedure. Interestingly, while translocated HiBiT-CagA increased up to an MOI 50 and then decreased (Figure 2a), the total HiBiT-CagA values increased with bacterial concentration in a linear fashion up to an MOI of 100 (Figure 2b), indicating that neither substrate nor LgBiT amounts were limiting, and that bacterial adhesion to target cells is not saturated in this range. When we calculated ratios...
between translocated CagA and total CagA, we obtained values between 3.5% and 12.5%, depending on the bacterial to target cell ratio (Figure 2c). Thus, *H. pylori* is able to translocate up to 12.5 ± 2% of its CagA content when present at one bacterial cell per target cell or less, but only lower fractions at higher concentrations, although the total amount of CagA per bacterial cell does not seem to be reduced at higher MOIs.

### 2.3 | Kinetics of CagA type IV secretion

Since the HiBiT translocation assay requires only the addition of substrate to the bacteria-target cell co-incubation mixture, we modified the protocol by adding the substrate directly at *t* = 0 in order to monitor translocation kinetics via luminescence generation in real-time. The results showed an increase of luminescence already after 10 min of infection with P12 [HiBiT-CagA], whereas no increase was detected with the ΔcagT mutant (Figure 3a). Luciferase activity increased for about 60 min and then reached a plateau at a level which was dependent on the bacteria-to-target cell ratio, with maximal levels at MOIs 13 and 25 (Figure S3a). However, the kinetics were similar for different MOIs, and attempts to spike in more substrate after 30 or 60 min did not result in higher luminescence values either (data not shown), suggesting that saturation is not due to substrate exhaustion. For unknown reasons, luminescence values were considerably lower for the real-time assay than for the end-point assay, and the sensitivity of the real-time assays was also lower in terms of the minimal MOI required for detection (Figure S3a). Because there was no signal accumulation in the assay, the HiBiT-CagA/HaloTag-LgBiT complexes were reasonably stable within the time frame used in our experiments (Figure S1b), we assumed that the luminescence signal at any given time point is roughly proportional to the amount of HiBiT-CagA translocated until this time point. Since luminescence curves in the HiBiT real-time assay show steep initial slopes (Figure 3a), we therefore concluded that *H. pylori* is able to rapidly inject substantial amounts of CagA within a short time, while injection quickly reaches saturation, at least under the conditions of our assays. At later time points, the curves thus indicate that not enough HiBiT-CagA can be delivered to AGS [LgBiT] cells to make up for the decay of functional luciferase complexes.

In order to translocate CagA, *H. pylori* has to establish target cell contacts that are productive for type IV secretion. For example, binding to CEACAM receptors at the target cell surface via the outer membrane protein HopQ represents such a productive interaction (Königer et al., 2016). To assess the influence of this interaction during early stages of translocation, we compared a P12 [HiBiT-CagA] strain with its isogenic hopQ deletion mutant. In line with previously reported data, the ΔhopQ mutant translocated over all about 50% less HiBiT-CagA than the parental strain (Figure 3b). Our real-time translocation assay further showed slightly different translocation kinetics, in which the ΔhopQ mutant seemed to reach half-maximal luminescence values later than the parental strain, or a complemented ΔhopQ mutant (Figure 3b), indicating that the interaction of HopQ with CEACAM receptors may facilitate faster translocation. Independent of adhesin-receptor interactions, faster cell contact may also be achieved mechanically by centrifugation of *H. pylori* onto the AGS cell layer. To examine this, we used the HiBiT-CagA real-time translocation assay to measure type IV secretion with and without prior centrifugation. Prior centrifugation indeed resulted in an earlier onset of CagA translocation, and the luminescence reached higher values as without centrifugation (Figure 3c). Interestingly, a flaA deletion mutant of the P12 [HiBiT-CagA] strain showed a reduced translocation efficiency as well, suggesting that flagellar motility enables the bacteria to contact cells more rapidly (Figure 3c). In support of this conclusion, centrifugation of the ΔflaA mutant onto the AGS cells fully rescued this defect and resulted in translocation rates similar to the wild-type under the same conditions.

As a different possibility to disturb efficient translocation, we examined the impact of presumptive inhibitors on CagA translocation. To do this, we started co-incubation of P12 [HiBiT-CagA] and AGS [LgBiT] cells, and added the inhibitors to the co-incubation mixture 20 min later. Under these conditions, adding the protein synthesis inhibitor erythromycin did not lead to dramatic changes in translocation kinetics, with a decrease in the luminescence signal seen only at later time points (Figure 3d), although erythromycin was shown to inhibit translocation when *H. pylori* is pretreated for 30 min (Schindele et al., 2016). Interestingly, however, administration of erythromycin resulted in a rapid decrease in bacterial viability (Figure S3b). In contrast, adding the anticancer drug cisplatin, which interferes with CagA translocation and with *H. pylori* viability by yet unknown mechanisms (Lettl et al., 2020), resulted in an almost immediate reduction of the CagA translocation rate (Figure 3d), but not in rapid killing of the bacteria (Figure S3b), supporting the previous conclusion that cisplatin acts rapidly and directly on the type IV secretion process.

### 2.4 | Requirement of protein unfolding for CagA translocation

Taking advantage of the high sensitivity of the HiBiT-CagA translocation assay, we next used it to address the impact of protein folding on CagA translocation. It is generally assumed that CagA has to be unfolded before passage through the secretion channel (Pattis et al., 2007), but experimental evidence for this has been lacking so far. To address this question, we generated N-terminal CagA fusions with the HiBiT tag and further passenger domains with different folding characteristics. First, we examined fusions with ubiquitin (Ub), which is known to fold rapidly and to impede protein translocation across membranes in its folded state, or with a ubiquitin variant (UbI3G, I13G) with decreased conformational stability that can be unfolded more easily (Johnson & Varshavsky, 1994) (Figure 4a). As a control, we generated HiBiT-GFP-CagA, reasoning that the rigid β-barrel structure of folded GFP should prevent translocation, as shown for other T4SS (Trotker & Waksman, 2018). In comparison
tyrosine phosphorylation was observed for HiBiT-UbI3G, I13G-CagA occurred after translocation. Furthermore, since no processing, but a clear luminescence signal (Figure 4c), this indicates that proteolysis of both HiBiT-Ub- CagA versions by immunoblotting after immunoprecipitation of tyrosine-phosphorylated proteins from infected AGS cells, although the signal was very weak for HiBiT-UbI3G, I13G-CagA (Figure S4a), this proteolysis step obviously depends on Ub folding, either suggesting that Ub is translocated in a folded state, or that it folds rapidly upon arrival in the AGS cell, and then gets recognized by a cellular protease, presumably a deubiquitinase.

To corroborate and extend these findings with an alternative approach, we generated a strain producing a fusion of the HiBiT tag together with murine dihydrofolate reductase (DHFR) to the N-terminus of CagA (Figure 5a). DHFR is a 22 kDa single-domain protein which folds rapidly and can be stabilized in its folded conformation by folate analogs such as methotrexate (Eilers & Schatz, 1988). The HiBiT-DHFR-CagA fusion protein was produced well by strain P12, but detection by HiBiT blotting showed partial processing and the release of low molecular-weight HiBiT-containing fragments (Figure S4b). Nevertheless, translocation of HiBiT-DHFR-CagA to AGS [LgBiT] cells could be detected with the HiBiT translocation assay, albeit at much lower rates than HiBiT-CagA (Figure S4c). Importantly, HiBiT-DHFR-CagA translocation was efficiently blocked by adding methotrexate in a dose-dependent manner, whereas methotrexate did not inhibit translocation of HiBiT-CagA lacking DHFR (Figure 5b). Using a phosphotyrosine immunoblot after immunoprecipitation from infected cells, as described above, we were able to detect a very weak phosphorylation of DHFR-CagA lacking the HiBiT tag (Figure S4a). This phosphorylation was not seen after infection in the presence of methotrexate, as expected, but we noted that the sensitivity of the phosphotyrosine assay was much lower than that of the HiBiT translocation assay (Figure 5b). Since methotrexate does not bind to...
unfolded DHFR, these results strongly suggested that protein folding takes place prior to introduction of the substrate into the type IV secretion channel, and that interfering with substrate unfolding prevents translocation. The possibility of blocking DHFR-CagA translocation by adding methotrexate enabled us to ask whether interference with unfolding of this substrate would lead to a general jamming of the type IV translocation machinery. Therefore, we next generated a strain which produces both HiBiT-CagA (from the recA locus) and DHFR-CagA (from the cagA locus), and confirmed by immunoblot analysis that both proteins were produced at similar levels (Figure 5c). In the HiBiT translocation assay, this strain showed strongly reduced levels of HiBiT-CagA translocation, as compared to a strain producing only HiBiT-CagA. Nevertheless, treatment with methotrexate to stabilize folded DHFR-CagA still caused a significant reduction in HiBiT-CagA translocation (Figure 5d). Collectively, this indicated that DHFR-CagA directly competes with HiBiT-CagA for entry into the translocation channel, and that unfolding-resistant substrates stall the translocation process.

3 | DISCUSSION

Translocation reporters that allow quantitative monitoring of substrate transport have facilitated the identification of effector proteins, and the analysis of molecular details of transport in different T4SS. For example, Cre recombinase (Lang et al., 2010; Vergunst et al., 2000), adenylate cyclase (Chen et al., 2004; Nagai et al., 2005), or β-lactamase fusions (de Felipe et al., 2008; Schindele et al., 2016) have been used to demonstrate type IV translocation of effector proteins, and to elucidate the nature of type IV secretion signals. NanoLuc luciferase or split-luciferase fusions have been employed as reporters to study Sec-dependent transport (Pereira et al., 2019), or type III secretion in Yersinia (Lindner et al., 2020) and Salmonella (Westerhausen et al., 2020). The advantage of the split-luciferase system used here is that the reporter tag is small and thus does not interfere with the secretion process, as confirmed by assessing CagA-dependent phenotypes, and that detection is very sensitive and does not require any lytic or other cumbersome post-infection treatment. The results of this study show that an efficient monitoring of type IV secretion by the H. pylori Cag system is achieved with both end-point and real-time protocols. Our data indicate that the luminescence signal is directly proportional to the amount of translocated HiBiT-CagA, as may be expected since there is no accumulation of reaction products that influence the readout signal. Furthermore, we were able to establish a protocol including a lysis step to obtain additional quantitative information about the fraction of bacterial effector protein that gets translocated. The only limitation of this reporter system is that it depends on LgBiT production...
in the target cell, which is more difficult to achieve in primary cells or in animal models.

Because of the low toxicity of the furimazine substrate, monitoring translocation in real time is possible over several hours. This enabled us for the first time to examine CagA translocation kinetics at early as well as later time points of co-incubation. Our data show that CagA gets translocated within only a few minutes after host cell contact, which also implies that the culture conditions used might facilitate a preassembly of secretion apparatus complexes prior to contact with target cells. Under experimental conditions which establish rapid contact formation, such as centrifugation of bacteria onto the cell layer, the initial translocation rates can even be increased. An earlier report suggested that flagellar motility may facilitate host cell interactions permissive for type IV secretion by providing faster contact, and that centrifugation of \textit{H. pylori} on epithelial cells may increase the interaction rate (Asakura et al., 2010). Both observations could be confirmed here with the HiBiT-CagA translocation assay. Deletion of the hopQ gene resulted in only slightly delayed translocation kinetics, but in lower overall translocation rates. This is in line with the previous conclusion that other ligand-receptor interactions which are conducive to type IV secretion may form between \textit{H. pylori} and certain target cells (Königer et al., 2016), but it also shows that these other putative routes to type IV secretion do not reach full efficiency, corroborating the finding that the HopQ-CEACAM interaction is necessary for full translocation rates (Zhao et al., 2018).

Generally, the kinetics of CagA translocation featured an initial burst of effector protein delivery, followed by a plateau phase after 60 to 80 min, in which newly translocated HiBiT-CagA is presumably just sufficient to compensate for the slow decay of active luciferase complexes. It is not clear whether CagA translocation rates are slowed down in this phase by the secretion system itself, or by a respective cellular response. However, the plateau phase was also observed when only low amounts of HiBiT-CagA were translocated (i.e., at lower MOI values), which does not only indicate that this plateau phase does not result from limited LgBiT or substrate levels, but also that it is not the amount of injected, intracellular CagA which regulates translocation efficiency. Since the total amount of HiBiT-CagA present in the infection mixture perfectly correlated with the number of bacteria used for infection, we could further exclude that adherence is a limiting factor for CagA translocation. This supports previous findings, in which pre-incubation of AGS cells with one \textit{H. pylori} strain had no impact on adherence of a second strain, even
at comparably high overall MOIs (Jiménez-Soto et al., 2013). In any case, only a minor fraction of the total bacterial CagA pool is finally translocated into target cells, which corroborates earlier conclusions obtained by quantification of CagA tyrosine phosphorylation (Jiménez-Soto & Haas, 2016). As concluded previously from experiments with protein biosynthesis inhibitors (Schindele et al., 2016), one reason for the limited amount of translocated protein might be that only newly synthesized CagA is in a translocation-competent state, for example, by interacting with the secretion chaperone CagF (Bonsor et al., 2013). In contrast to this, a rapid exhaustion of intrabacterial effector protein pools was often observed in type III secretion systems of Salmonella enterica, Shigella flexneri, or enteropathogenic Escherichia coli, where a similar immediate onset of effector protein delivery upon target cell contact has been described as well (Enninga et al., 2005; Mills et al., 2008; Schlumberger et al., 2005). For example, the pool of the S. enterica effector protein SipA was found to be quickly translocated within 2 to 10 min, clearly indicating limited amounts of this effector (Schlumberger et al., 2005). Furthermore, only a minor fraction (26%) of the S. enterica population was found to express sipA under in vitro conditions, while flow cytometry experiments performed here with GFP-CagA-producing H. pylori (data not shown) suggest that cagA is constitutively expressed by all bacterial cells. In addition, the MOI during infection, and thus the bacterial density, did not impact on the total amount of HiBiT-CagA synthesized. Therefore, it remains unclear why H. pylori synthesizes such high amounts of CagA in vitro, but also in vivo, albeit at slightly lower levels (Avilés-Jiménez et al., 2012; Boonjakuakul et al., 2005). To our knowledge, there is currently no indication for an additional function of (non-translocated) CagA in the bacterial cell. The inverse relationship between the number of adherent bacteria and the fraction of translocated CagA indicates an optimum at about one bacterium per epithelial cell, which might be in a similar range as bacterial densities observed in gastric glands of colonized mice (Fung et al., 2019). At higher bacterial densities, the total amount of translocated CagA would reach a maximum and then decrease again (Figures 2a, S3a), possibly to prevent inflicting too much damage. As CagA has been described to be involved in acquisition of nutrients for microcolony formation (Tan et al., 2011), it is also conceivable that CagA translocation becomes dispensable at higher colonization densities.

It is well-established that T4SS comprise a wide variety of transported substrates or secretion apparatus architectures (Christie et al., 2014), and substantial variations exist for their molecular secretion mechanisms as well. Nevertheless, most T4SS probably secrete their substrates directly from the cytoplasm, without periplasmic intermediates, and are believed to depend on substrate unfolding prior to transport (Christie et al., 2014). Indeed, substrates of different T4SS show strongly reduced transport rates, or no transport at all, when they are fused to rapidly folding protein domains such as Ub or DHFR (Amyot et al., 2013; Trokter & Waksman, 2018). We show here that the Cag system exhibits strongly reduced translocation rates for a DHFR fusion protein, and no translocation of a GFP fusion, but is still able to transport Ub-CagA fusions at almost wild-type rates (Figure 4e). Thus, at least transport of the small Ub domain by the Cag system is markedly different from the situation in the R388 conjugation system, where addition of folded Ub to a fusion of Cre recombinase with the relaxase TrwC resulted in a decrease of transport efficiency by three orders of magnitude (Trokter & Waksman, 2018), or from type III secretion systems of Yersinia enterocolitica or S. enterica, where corresponding fusions were not detectably secreted (Lee & Schneewind, 2002; Radics et al., 2014). Our data therefore suggest that Ub is either translocated in a folded state within the HiBiT-Ub-CagA fusion, or that the secretion machinery is able to provide enough energy to unfold Ub (but not GFP or DHFR bound to methotrexate) prior to translocation. Although we cannot completely rule out the first possibility, we favor the alternative explanation that folded Ub is not stable enough to resist the substrate unfolding activity of the Cag secretion system, for several reasons:

First, although detailed structural analysis of whole Cag secretion apparatus complexes by cryo-electron tomography (Chang et al., 2018; Hu et al., 2019), or of isolated outer membrane-associated core complex particles by cryo-electron microscopy (Chung et al., 2019; Sheedlo et al., 2020) has been performed, there is currently no structural evidence that the translocation channel is able to accommodate folded substrates. Second, the difference between the strongly reduced, but still substantial translocation rates of HiBiT-DHFR-CagA, and the complete incapacity of HiBiT-GFP-CagA to be translocated, is most likely not due to the (minor) size differences of the corresponding folded domains, but probably rather due to a general resistance of GFP to become unfolded, as shown for the Salmonella SPI-1 type III secretion system (Akedo & Galan, 2005). Indeed, GFP fusions have recently been used to trap a type III-secreted substrate inside the translocation channel, where it could be visualized in an unfolded state (Miletic et al., 2021). Our interpretation of the differences in translocation rates is, thus, that the DHFR and Ub domains require less energy for the unfolding process. Third, the inhibitory activity of methotrexate observed here indicates on the one hand that the DHFR domain is actually folded in H. pylori, since methotrexate would not bind otherwise. On the other hand, it shows that stabilization of DHFR folding prevents translocation of HiBiT-DHFR-CagA, an effect that can hardly be explained by assuming transport of folded domains. These observations are also in contrast to the Yersinia Ysc type III secretion system, where a YopE-DHFR fusion can only be secreted when it is kept in an unfolded state by the SycE chaperone (Feldman et al., 2002). Nevertheless, we assume that unfolding of CagA itself is facilitated by its interaction with the secretion chaperone CagF (Pattis et al., 2007), which is able to bind to several CagA domains (Bonsor et al., 2013), but probably not to DHFR. Finally, our observation that co-production of HiBiT-CagA and DHFR-CagA led to more severe inhibition of HiBiT-DHFR-CagA, an effect that can hardly be explained by assuming transport of folded domains. These observations are also in contrast to the Yersinia Ysc type III secretion system, where a YopE-DHFR fusion can only be secreted when it is kept in an unfolded state by the SycE chaperone (Feldman et al., 2002). Nevertheless, we assume that unfolding of CagA itself is facilitated by its interaction with the secretion chaperone CagF (Pattis et al., 2007), which is able to bind to several CagA domains (Bonsor et al., 2013), but probably not to DHFR. Finally, our observation that co-production of HiBiT-CagA and DHFR-CagA led to more severe inhibition of HiBiT-DHFR-CagA translocation in the presence of methotrexate, also supports the assumption that substrate unfolding is required. In this notion, DHFR-CagA would be introduced into the secretion apparatus, which would be unable to process the fusion protein in the presence of methotrexate, due to stabilized folding of DHFR, resulting in physical obstruction of the secretion channel. This would also imply that
CagA is introduced with its C-terminus first into the secretion channel, as speculated previously (Woon et al., 2013), and would be consistent with the notion that the CagA C-terminus contains the major, although not the only, type IV secretion signal (Hohlfeld et al., 2006; Schindele et al., 2016).

In conclusion, the HiBiT-CagA translocation assay developed here is a powerful tool to explore mechanistic details of type IV secretion by quantitative measurements with a high sensitivity, and optionally in real-time experimental setups. As a complement to the previously described TEM-1-CagA translocation assay (Schindele et al., 2016), it has the potential to enable advanced functional studies of the widespread family of bacterial type IV protein transporters.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains, cells and infection conditions

*H. pylori* strains were grown on GC agar plates (Oxoid) supplemented with vitamin mix (1%) and horse serum (8%) (serum agar plates) and cultured for 16–60 hr in a microaerobic atmosphere (85% N₂, 10% CO₂, 5% O₂) at 37°C. DNA was introduced into *H. pylori* strains by transformation, as described previously (Haas et al., 1993). *H. pylori* transformants were selected on serum plates containing 6 mg/L chloramphenicol, 8 mg/L kanamycin, 10 mg/L erythromycin, or 250 mg/L streptomycin.

4.2 | Plasmid constructs

All PCR amplification, cloning and DNA analysis procedures were performed according to standard protocols (Sambrook & Russell, 2001). Gene fusions encoding N-terminally tagged CagA variants were introduced in the *cagA* locus via a two-step protocol using the rpsL-erm counterselection system (Rohrer et al., 2012). Briefly, replacement of a 5’ *cagA* region by an rpsL-erm cassette was achieved via transformation of strain P12 (Strep R) with plasmid pWS556, and tagged variants were reintroduced by homologous recombination and selection on streptomycin resistance, using the respective plasmids (see below). Plasmid pWS556 is an EcoRI deletion derivative of plasmid pWS403 (Schindele et al., 2016), in which the 5’ *cagA* region (bases 1-770) was removed by inverse PCR with primers WS242 and WS545 (Table S1), and replaced with an rpsL-erm cassette. The HiBiT-cagA′-encoding plasmid pCL3 was generated by blunt-end ligation of an inverse PCR (primers CL67/CL68) from pCL19. For plasmids pCL21 (HiBiT-gfp-cagA′) and pCL23 (HiBiT-dhfr-cagA′), the SalI/BamHI fragment of pCL19 was replaced by gfp amplified from pWS130 (Hohlfeld et al., 2006) using primers CL69 and CL70, or by dhfr amplified from plasmid pCMV6-Kan/Neo-dhfr (Origene, MR201752) using primers CL71/CL72, respectively. The dhfr-cagA′ encoding plasmid pCL24 was obtained by religation of a PCR product obtained with WS242/CL71 (XhoI/Sall), using pCL23 as template. Full length HiBiT-cagA was amplified from genomic DNA of P12 [HiBiT-CagA] using primers JP28 and JP67, and ligated into the recA integration vector pJP99 (Püls et al., 2002) to generate plasmid pCL27. To generate the corresponding isogenic deletion mutants, plasmids pJP95 ΔcagT [Fischer et al., 2001], pFS10 Δ(hopQ, Bonsor et al., 2018]), and pRH121 ΔΔlpaA, [Haas et al., 1993]) were used. For complementation of the ΔhopQ mutant, plasmid pWS658 (Bonsor et al., 2018) was used.

4.3 | Cell lines

AGS cells were cultivated under standard conditions in 75 cm² tissue culture flasks (BD Falcon) and subcultivated every 2–3 days in 6-well, 24-well (tissue culture treated, Costar, Corning Inc.), or 96-well microtiter plates (black, transparent bottom, tissue cultured treated, 4titude®, as described previously (Fischer et al., 2001). For microscopy, cells were seeded in IBIDI 8-well µ-slides. To generate the HaloTag-LgBiT-producing reporter cell line, AGS cells were stably transfected with plasmid pCS1956B02 (Promega), as described previously (Zhao et al., 2018). To obtain a cell line derived from a single cell clone, transfected cells were stained overnight for HaloTag synthesis (R110Direct ligand G3221, Promega), and subsequently sorted (BD FACSAria III, HaloTag-liqBiT) according to the manufacturer’s protocol, and examination of samples with a Leica TCS SP5 confocal microscope. Image processing was performed with ImageJ 1.48v. (National Institutes of Health). AGS [LgBiT] cells were cultivated in the same way as AGS cells, except that the medium was supplemented with 0.5 mg/ml hygromycin B (Thermo Fisher). On the day before infection with *H. pylori*, this medium was changed to RPMI/FCS only.

4.4 | Antibodies, SDS-PAGE and immunoblotting

Rabbit polyclonal antisera AK257, AK299, and AK270 directed against CagA, the CagA EPIYA region, and CagT, respectively, have been described previously (Fischer et al., 2001; Schindele et al., 2016). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was performed as described previously (Fischer et al., 2001). For the development of immunoblots, polystyrene dienofluoride (PVDF) filters were blocked with 5% non-fat milk powder in TBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl), 0.1% (v/v) Tween 20 (TBS-T), and incubated with the respective
bands were visualized using a chemiluminescence imager.

...with 1x Nano-Glo® blotting buffer containing recombinant LgBiT membranes were incubated overnight at 4°C. To monitor HiBiT-CagA translocation into AGS [LgBiT] cells, the Nano-Glo® Live Cell Assay System (N2410, Promega) was adapted and cell debris were removed by centrifugation (18,000 g, 10 min, 4°C). Protein G agarose was pelleted and washed three times with冷 RIPA buffer supplemented with protease inhibitors (see PBS*) and lysed via sonication. Unbroken cells and cell debris were removed by centrifugation (18,000g, 10 min, 4°C). An aliquot of the supernatant was mixed with the respective volume of 2x SDS sample buffer and boiled for 10 min. The remaining supernatant was incubated overnight at 4°C with the phosphotyrosine specific antibody PY99 while rotating (1:250). The next day, protein G agarose was added (50 µl/450 µl sample) and incubation was continued for 2 hr at 4°C. Protein G agarose was pelleted and washed three times with cold RIPA* (18,000g, 30 s, 4°C). Finally, 50 µl of 2x SDS sample buffer were added and samples were boiled for 10 min.

AGS cells seeded in 75 cm² cell culture flasks were co-incubated with the respective H. pylori strains and subsequently samples for phosphotyrosine immunoblotting were prepared as described (Fischer et al., 2001). Briefly, cells were infected with the bacteria at an MOI of 100 for 4 hr at 37°C, 5% CO₂, washed twice with cold PBS and suspended in PBS containing 1 mM Na₃VO₄, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin (PBS*). Cells with adherent bacteria were collected by centrifugation and resuspended in SDS-PAGE sample solution. Tyrosine-phosphorylated proteins were analyzed by immunoblotting with the phosphotyrosine antibody PY99 (Santa Cruz Biotechnologies). To analyze poorly translocated CagA variants, AGS cells seeded in 75 cm² cell culture flasks were co-incubated with the respective strains at an MOI of 60 as described above, but bacteria were pre-incubated for 2 hr in PBS/FCS. Optionally, 100 µM methotrexate was added during the last 30 min of pre-incubation. Samples were washed with PBS*, and cells with adherent bacteria were harvested by centrifugation, and resuspended in 500 µl radioimmunoprecipitation (RIPA) buffer supplemented with protease inhibitors (see PBS*) and lysed via sonication. Unbroken cells and cell debris were removed by centrifugation (18,000g, 10 min, 4°C). An aliquot of the supernatant was mixed with the respective volume of 2x SDS sample buffer and boiled for 10 min. The remaining supernatant was incubated overnight at 4°C with the phosphotyrosine specific antibody PY99 while rotating (1:250). The next day, protein G agarose was added (50 µl/450 µl sample) and incubation was continued for 2 hr at 4°C. Protein G agarose was pelleted and washed three times with cold RIPA* (18,000g, 30 s, 4°C). Finally, 50 µl of 2x SDS sample buffer were added and samples were boiled for 10 min.

Translocation and HiBiT quantification assays

To monitor HiBiT-CagA translocation into AGS [LgBiT] cells, the Nano-Glo® Live Cell Assay System (N2011, Promega) was adapted in two different protocols. For monitoring translocation in real-time (HiBiT Live Assay), bacteria expressing the HiBiT-CagA fusion protein were diluted to an OD₅₅₀ of 0.004 to 0.4 (depending on the MOI to be used) in PBS/10% FCS and preincubated for 2 hr at 37°C, 10% CO₂. Next, AGS [LgBiT] cells seeded in a 96-well plate (4titude) were infected with 40 µl preculture, and 10 µl 5x luciferase substrate (9.5 µl assay buffer +0.5 µl furimazine substrate) were added. Luminescence measurements at 470 nm (20 nm bandwidth, 10 s integration time, gain 4,095, bottom optics) were started immediately and detected every 3–5 min for up to 2.5 hr in a prewarmed Claristat plate reader with CO₂ levels adjusted to 5% using an atmospheric control unit. Optionally, bacteria were brought into contact with target cells by centrifugation (5 min, 2,500g) of 96-well plates immediately after starting the co-incubation. To examine the impact of inhibitors on HiBiT-CagA translocation, the measurement was shortly paused after 20–30 min of infection to add the respective compounds, or DMSO, and the measurement was continued.

To measure the amount of translocated HiBiT-CagA, HiBiT-CagA-expressing H. pylori strains were pre-cultured in PBS/10% FCS as described above. Optionally, bacteria were treated with the indicated concentrations of methotrexate during the last 30 min of pre-incubation. AGS [LgBiT] cells seeded in a 96-well plate (4titude) were infected with 200 µl preculture, and incubated at 37°C, 5% CO₂ for 2.5 hr. Then, supernatants including unbound bacteria were discarded, and cells were loaded with 40 µl PBS/FCS and 10 µl 5x luciferase substrate mix, as above. After 10 min incubation, luminescence was measured as described above. The amount of translocated HiBiT-CagA was calculated after correction for the background signal as luminescence counts (ΔRLU), or as percentage in relation to the P12 [HiBiT-CagA] control. Optionally, after quantifying translocated CagA, the total amount of CagA present in the AGS [LgBiT] cells and adherent bacteria was analyzed. To do so, 50 µl cold RIPA buffer was added to each well, and samples were lysed at 4°C for 5–10 min. Next, 20 µl lysate were mixed with 20 µl of a Master Mix containing 2 µl 10x assay buffer, 0.2 µl LgBiT, and 0.1 µl furimazine substrate, in black flat-bottom 96-well plates (Corning). Luminescence was recorded, as described above, using top optics. For the blank value, luminescence of uninfected cells was measured. "Total HiBiT-CagA" values were calculated considering a dilution factor of five from the original sample.

Killing assay

AGS cells seeded in 12 well culture plates were infected with 800 µl H. pylori P12 in PBS/FCS at an OD₅₅₀ of 0.1. After 30 min of incubation at 37°C, 5% CO₂, inhibitors or DMSO were added at the indicated concentrations, and co-incubation was continued. Cells with adherent bacteria were scraped off at 0, 30, 60, or 120 min after compound administration, and aliquots of the infection mixture were spread on nonselective serum agar plates, and incubated to determine total numbers of viable bacteria.
4.8 | Statistical analysis

Unless indicated otherwise, quantitative data shown are means with standard deviations of at least three independent experiments. In case of the real-time HiBiT-CagA translocation assay, representative results with means and standard deviations derived from two technical replicates are depicted. Statistical analysis was performed using the GraphPad Prism 5 software. The significance of differences was determined using One-way ANOVA with the indicated post-hoc tests. Linear regression analysis was calculated considering individual replicate values of each Y point. The goodness of fit is indicated as R².

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Conception and design of the study: RH and WF. Acquisition of data: CL. Analysis and interpretation of data: CL and WF. Writing of the manuscript: CL and WF.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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Additional Supporting Information may be found online in the Supporting Information section.

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