





# Salicylidene Acylhydrazides and Hydroxyquinolines Act as Inhibitors of Type Three Secretion Systems in *Pseudomonas aeruginosa* by Distinct Mechanisms

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**ABSTRACT** Type 3 secretion systems (T3SSs) are major virulence factors in Gram-negative bacteria. *Pseudomonas aeruginosa* expresses two T3SSs, namely, an injectisome (iT3SS) translocating effector proteins in the host cell cytosol and a flagellum (fT3SS) ensuring bacterial motility. Inhibiting these systems is an appealing therapeutic strategy for acute infections. This study examines the protective effects of the salicylidene acylhydrazide INP0341 and of the hydroxyquinoline INP1750 (previously described as T3SS inhibitors in other species) toward cytotoxic effects of *P. aeruginosa* *in vitro*. Both compounds reduced cell necrosis and inflammasome activation induced by reference strains or clinical isolates expressing T3SS toxins or only the translocation apparatus. INP0341 inhibited iT3SS transcriptional activation, including in strains with constitutive iT3SS expression, and reduced the total expression of toxins, suggesting it targets iT3SS gene transcription. INP1750 inhibited toxin secretion and flagellar motility and impaired the activity of the YscN ATPase from *Yersinia pseudotuberculosis* (homologous to the ATPase present in the basal body of *P. aeruginosa* iT3SS and fT3SS), suggesting that it rather targets a T3SS core constituent with high homology among iT3SS and fT3SS. This mode of action is similar to that previously described for INP1855, another hydroxyquinoline, against *P. aeruginosa*. Thus, although acting by different mechanisms, INP0341 and INP1750 appear as useful inhibitors of the virulence of *P. aeruginosa*. Hydroxyquinolines may have a broader spectrum of activity by the fact they act upon two virulence factors (iT3SS and fT3SS).

**KEYWORDS** NLR4 inflammasome, *Pseudomonas aeruginosa*, flagella, inhibitors, type three secretion system, virulence

I ncreasing resistance of clinical isolates of *Pseudomonas aeruginosa* to current recommended antipseudomonal antibiotics is associated with poor clinical outcome and increased mortality (1–3), prompting the search for innovative therapeutic strategies. However, very few novel antipseudomonal agents are currently under development (4). This is partly due to difficulties in identifying and developing new molecules active on still unexploited targets, but it is also due to the low permeability of the outer membrane of *P. aeruginosa* (combining active efflux or poor porin permeability), which prevents many active molecules from reaching targets located in the bacterial cytosol or in the periplasmic space (5). In this context, innovative therapeutic strategies are needed (6), among which those aiming at reducing bacterial virulence are particularly appealing, because they reduce morbidity and improve antibacterial immune re-

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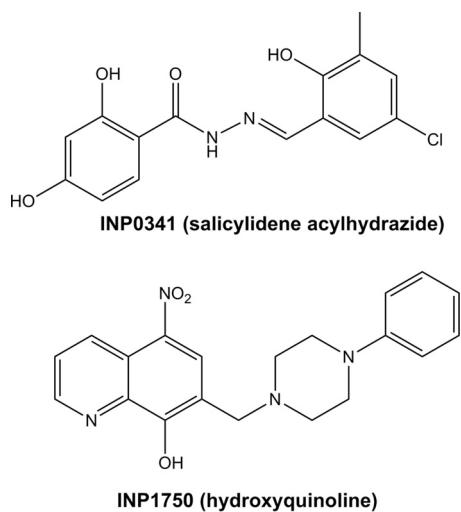
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sponses while preserving commensal flora (7). These strategies also remain compatible with antibiotic usage and are expected to have a lower risk of selecting resistance, since they only disarm bacteria, leaving the task of killing them to the immune system (8, 9).

Among other virulence factors, *P. aeruginosa* expresses two type 3 secretion systems (T3SSs), namely, an injectisome (referred to as iT3SS) translocating effector proteins directly in the host cell cytosol and a flagellum (designated fT3SS) ensuring bacterial motility (10). iT3SS expression is associated with poor clinical outcomes and high morbidity in acute infections (3, 11, 12). The system consists in a syringe-like machine injecting bacterial effector proteins from the bacterial cytosol directly into the host cell by the concerted action of a secretion apparatus (comprising among others the protein PscF forming the needle filament) and a translocon (proteins PopB, PopD, and PcrV; see Fig. S1 in the supplemental material for a schematic view). Among translocated proteins, effectors encoded by the iT3SS operon include ExoU (a phospholipase A<sub>2</sub> causing rapid cell death by altering membrane integrity), ExoS and ExoT (causing disruption of actin filaments and subsequent prevention of phagocytosis, as well as cytotoxicity), and ExoY (contributing to actin cytoskeleton disorganization) (13). Importantly, other proteins can be translocated through the iT3SS, such as FliC (monomeric subunit of flagellum) (14, 15). FliC can cause host cell damage independently of effectors, notably by activating the intracellular cytosolic sensor NLRC4 (NLR family, CARD domain containing 4) inflammasome resulting in pyroptosis (16) and in liberation of active forms of interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-18 (see reference 17 for a review). Animal models of acute pulmonary infections showed that the activation of this cascade contributes to increased lung injury and impaired *P. aeruginosa* clearance by reducing IL-17 signaling and antimicrobial peptide production (18, 19), the deleterious consequences of which for the host remain controversial (16). A third type of protein excreted by iT3SS is ExsE, a negative regulator of iT3SS transcription (20). It is secreted out of the bacteria under inducing conditions and liberates the transcription activator ExsA via a complex regulatory cascade, which then activates iT3SS gene transcription (21; see also reference 22 for a schematic view of this process). *In vitro*, iT3SS transcription is activated by contact with eukaryotic cells, low-Ca<sup>2+</sup> medium, or the presence of serum (23–26).

fT3SS is also considered to be a virulence factor that is required for bacterial motility but also for adhesion to the surfaces of host cells, the dispersal of biofilms, or initiation of the inflammatory response (27). It consists of a basal body anchored in the membranes and comprises an export apparatus delivering flagellin subunits into a channel, from which they diffuse and assemble to form the flagellum. iT3SS and fT3SS share a similar general structure. Their assembly is dependent upon a T3SS located in the inner membrane and occurs according to a similar process. Proteins directly involved in the secretion activity of iT3SS and the export activity of fT3SS share a high degree of similarity (28).

On these bases, inhibiting T3SS appears as an attractive therapeutic strategy (29, 30). At this time, two main types of T3SS inhibitors have been investigated against *P. aeruginosa* (see reference 22 for a review). The first ones are therapeutic antibodies blocking PcrV protein or hybrid antibodies targeting both the PcrV protein and the Psl exopolysaccharide simultaneously. The second type of inhibitors consists of small molecules identified by *in vitro* screening of libraries of natural or synthetic compounds. However, biological evaluation of the pharmacological effect of these compounds is often limited to the demonstration of a reduction in cytotoxicity, while their effects on inflammasome activation, a key element in the host response to infection, have not been explored. Among them, two chemical classes have been identified as inhibitors of iT3SS transcription using screening-based approaches with *Yersinia pseudotuberculosis*, namely, the salicylidene acylhydrazides and the hydroxyquinolines. They have also proven active against other Gram-negative bacterial species such as *Chlamydia*, *Shigella*, and *Salmonella* spp. (31–34). Based on the high homology among iT3SS from Gram-negative bacteria, we undertook to investigate their activity against *P. aeruginosa*. In a previous report (35), we showed that the hydroxyquinoline INP1855 reduces



**FIG 1** Chemical structure of the inhibitors used in this study.

the virulence of *P. aeruginosa* *in vitro* and *in vivo* (acute model of pulmonary infection) by inhibiting both the iT3SS and the fT3SS. This impairs the secretion of effectors and the flagellar motility, as well as the activity of a purified ATPase (homologous to that present in the basal core of these systems), protecting eukaryotic cells from necrosis and inflammasome activation and restoring bacterial phagocytosis.

In the present work, we selected a representative compound in each of these two chemical classes in order to compare their antipseudomonal activity. Among salicylidene acylhydrazides, we chose INP0341 (34, 36), which proved more potent against *P. aeruginosa* than other derivatives in the family (INP0010 and INP0406 [33, 37]) during a preliminary screening. Among hydroxyquinolines, we selected INP1750 (32), which differs from the previously studied INP1855 by the absence of fluorine (frequently used for pharmacomodulation) on the phenylpiperazine substituent of the hydroxyquinoline. In brief, we show that both INP0341 and INP1750 (Fig. 1) protect eukaryotic cells against cytotoxicity mediated by toxins and by inflammasome activation. These protective effects were observed against laboratory strains and clinical isolates. Interestingly, in spite of the similarity of their protective effects on the host cells, we show that they act by different mechanisms on bacteria.

## RESULTS

### Evaluation of INP0341 and INP1750 toxicity for bacteria and eukaryotic cells.

In preliminary experiments, we checked whether INPs exerted nonspecific toxic effects on bacteria and eukaryotic cells exposed to a broad range of INP concentrations (see Fig. S2 in the supplemental material). Toxicity toward bacteria was ruled out by demonstrating that INPs did not modify the growth rate of the clinical strain CHA at concentrations up to 250  $\mu\text{M}$ . Toxicity toward eukaryotic cells was determined by measuring the release of the cytosolic enzyme lactate dehydrogenase in the culture medium of THP-1 cells and A549 lung epithelial cells incubated for 5 and 7 h, respectively, with increasing inhibitor concentrations. These incubation times were selected based on preliminary experiments showing that the cytotoxic effects of CHA were dependent on the iT3SS under these conditions (35). No significant release of lactate dehydrogenase was observed from both cell types for concentrations of  $\leq 100 \mu\text{M}$  (INP0341) or  $\leq 80 \mu\text{M}$  (INP1750). Further experiments were therefore performed with concentrations of  $\leq 80 \mu\text{M}$ .

**Influence of INP0341 and INP1750 on iT3SS-mediated cytotoxicity and bacterial internalization.** We then studied the effect of INP0341 and INP1750 on bacterial interactions with eukaryotic cells, focusing first on iT3SS-induced cytotoxicity. In a first set of experiments, the effect of INPs on CHA-induced cytotoxicity was evaluated after

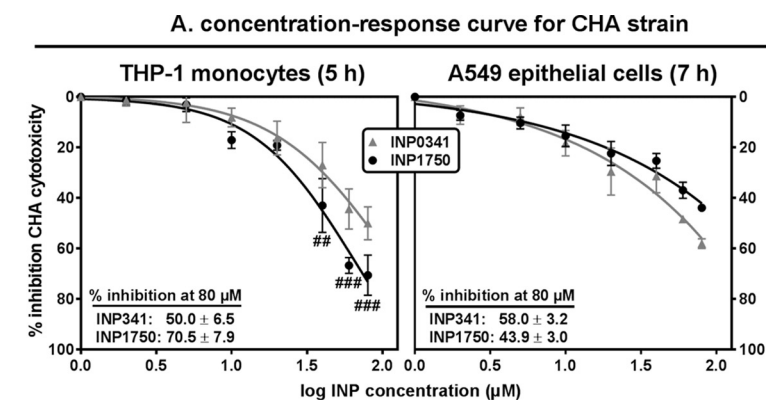
5 h (THP-1 monocytes) or 7 h (A549 epithelial cells) of incubation with 10 bacteria/host cell and increasing, noncytotoxic concentrations of INPs (Fig. 2A). The two inhibitors decreased the cytotoxicity of CHA in a concentration-dependent manner in both cell types. INP0341 was equipotent toward both cell lines. INP1750 was more potent toward THP-1 cells than A549 cells at concentrations of  $\geq 40 \mu\text{M}$ ; it was also significantly more active than INP0341 over the same concentration range in THP-1 cells. The study was then extended to compare the effect of INP0341 and INP1750 at  $80 \mu\text{M}$  on the cytotoxic effects of strains CHA, PA103, and selected mutants thereof (Fig. 2B). As previously described (35), CHA, PA103, and their mutants constitutively expressing the iT3SS (PA103 $\Delta\text{exsD}$  and PA103 $\Delta\text{exsE}$ ) were toxic for both cell lines, with PA103 causing a higher lactate dehydrogenase (LDH) release than CHA. Mutants with no expression of toxins (CHA $\Delta\text{STY}$  and PA103 $\Delta\text{UT}$ ) were selectively toxic for THP-1 cells, while mutants with no expression of the translocation apparatus (CHA $\Delta\text{popBD}$  and PA103 $\Delta\text{pcrV}$ ) or of the iT3SS global regulator (CHA $\Delta\text{exsA}$  and PA103 $\Delta\text{exsA}$ ) were not cytotoxic, demonstrating that the expression of iT3SS is responsible for the cytotoxicity observed in our experimental conditions. INP0341 and INP1750 impaired LDH release induced by all cytotoxic strains in both cell lines, with INP1750 being slightly more active than INP0341 in THP-1 cells.

We and others previously showed that CHA $\Delta\text{STY}$  or PA103 $\Delta\text{UT}$  (expressing the translocation apparatus but not the toxins) were selectively toxic for phagocytic cells by activating NLR4 inflammasome cascade (16, 18, 35, 38). This effect follows the injection of flagellin (14, 15), rod or needle proteins (39, 40), or pilin (41) in the host cell cytosol via the iT3SS. NLR4 inflammasome activation results in the cleavage of procaspase-1 into active caspase-1, which itself causes the maturation and release of IL-1 $\beta$  and IL-18, inducing cell death by pyroptosis (16). We therefore measured the release of IL-1 $\beta$  in the supernatant of THP-1 monocytes incubated for 5 h with different mutants of CHA and of PA103 in the presence of  $80 \mu\text{M}$  INPs or their vehicle (Fig. 2C). Nigericin was used in parallel as an inducer of the NLRP3 inflammasome (42) to test for the specificity of the effects of INPs. CHA, CHA $\Delta\text{STY}$ , and PA103 $\Delta\text{UT}$  strains caused a marked release of IL-1 $\beta$  that was significantly reduced by INP0341 and INP1750. Nigericin also increased IL-1 $\beta$  levels in the culture medium, but INPs did not reverse this effect. The other strains (including PA103) caused a smaller release of IL-1 $\beta$  (as previously described and discussed [35]) that was not prevented by INPs. Tumor necrosis factor alpha (TNF- $\alpha$ ) release was measured in parallel as a control, its release being independent of inflammasome activation. All strains (whether expressing the iT3SS or not), as well as the combination of LPS and nigericin, induced the release of TNF- $\alpha$  release, but this effect was not prevented by INPs (see Fig. S3 in the supplemental material).

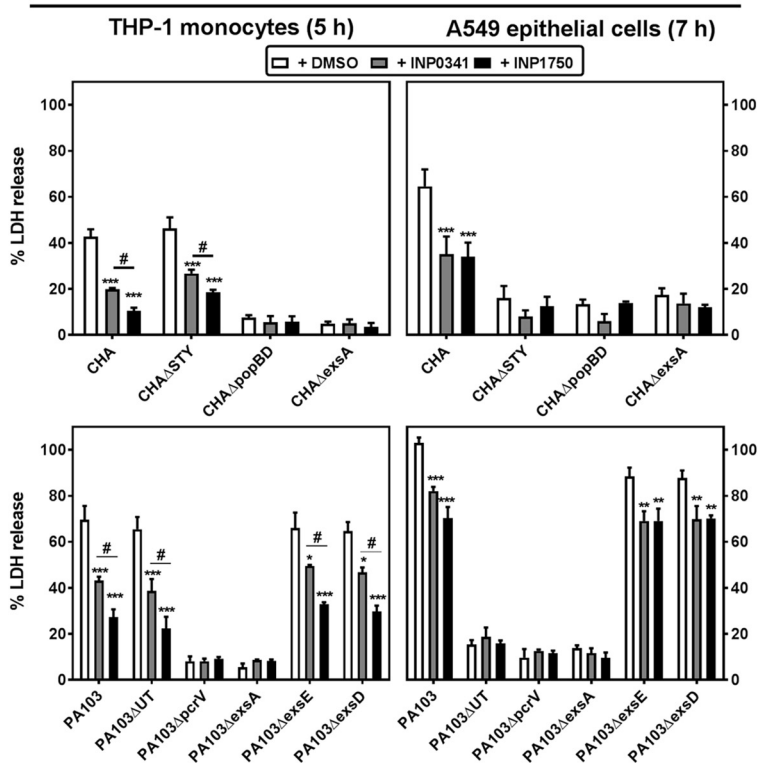
ExoS and ExoT inhibit bacterial internalization in eukaryotic cells by disorganizing actin microfilaments (43, 44). We therefore examined the influence of INPs on the internalization of CHA, using the aminoglycoside protection assay and focusing on phagocytic cells, which take up bacteria more avidly than epithelial cells. Bacteria were incubated during 3 h with  $80 \mu\text{M}$  INP0341 or INP1750 in broth, pelleted, and incubated with THP-1 monocytes in the presence of inhibitors during 2 h; the phagocytosed bacteria were then quantified by CFU counting. INP0341 and INP1750 significantly increased (2.6- and 2.8-fold, respectively) CHA internalization within THP-1 monocytes (see Fig. S4 in the supplemental material).

Thus, the two inhibitors reduced the cytotoxicity of strains expressing toxins in both phagocytic and epithelial cells and reduced the toxin inhibitory effect on bacterial internalization by phagocytes. In addition, they prevented the development of toxicity mediated by inflammasome activation in THP-1 cells, including for strains expressing the translocation apparatus but no toxins.

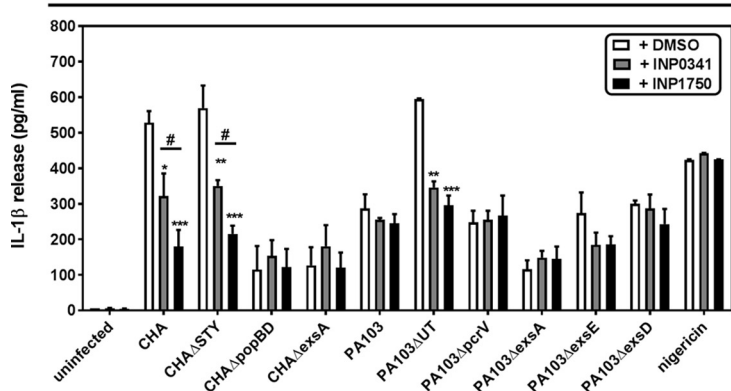
**Influence of INP0341 and INP1750 on iT3SS transcription.** We then examined the effect of INP0341 and INP1750 at increasing concentrations on iT3SS transcription, using a bioluminescent reporter strain (CHA pC::lux). Bacteria were incubated in the



**B. Effect of INP0341 and INP1750 on T3SS dependent-cytotoxicity in CHA, PA103 and their mutants**



**C. IL-1 $\beta$  secretion by THP-1 cells**



**FIG 2** Influence of INP0341 and INP1750 on iT3SS-mediated effects in phagocytic cells and epithelial cells. (A) Inhibition of CHA cytotoxicity by INP0341 and INP1750. Cell viability was assessed by measuring (Continued on next page)

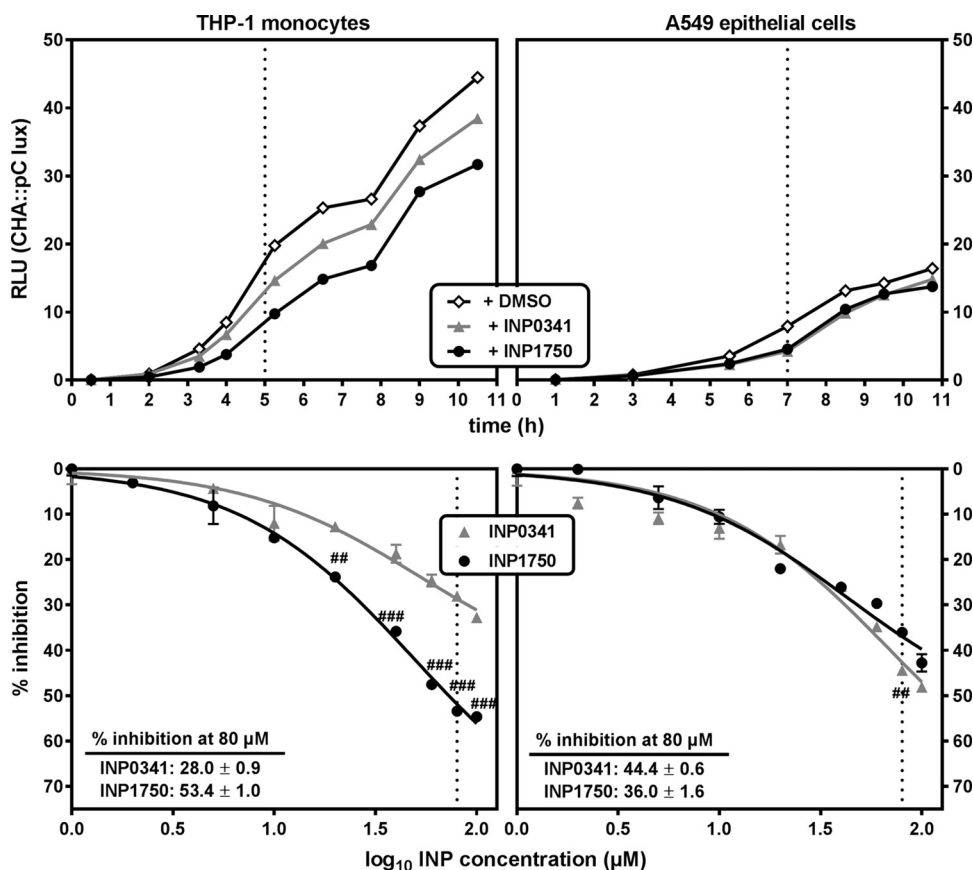
presence of THP-1 monocytes or A549 epithelial cells, *iT3SS* transcription being activated by contact with the eukaryotic cells. As shown in Fig. 3, *iT3SS* transcription was greater in the presence of THP-1 cells than in the presence of A549 cells. In the presence of THP-1 cells, INP1750 was a more potent inhibitor of transcription than INP0341 (53 versus 28% inhibition after 5 h at 80  $\mu$ M, respectively; there was a significant difference among them for concentrations  $\geq 20$   $\mu$ M). Conversely, no statistically significant difference was observed between the two inhibitors in the presence of A549 cells (36 to 44% reduction in the bioluminescence signal after 7 h at a concentration of 80  $\mu$ M).

In addition to contact with eukaryotic cells, media depleted in  $\text{Ca}^{2+}$  (21) or enriched in serum (24, 25) also activate *iT3SS* transcription. Since THP-1 cells and A549 cells were cultivated in different media (RPMI 1640 and Dulbecco modified Eagle medium [DMEM], respectively), we examined the influence of the medium composition on *iT3SS* transcription and its inhibition by INPs used at a fixed concentration of 80  $\mu$ M. The experiments illustrated in Fig. 3 were first performed in the absence of cells yielding similar results (see Fig. S5 in the supplemental material), suggesting that differences in culture media composition could by themselves explain the variation in *iT3SS* transcription levels observed in the absence of inhibitors between cultures in RPMI 1640 and in DMEM. Since RPMI 1640 contains less  $\text{Ca}^{2+}$  than DMEM, we supplemented RPMI 1640 with increasing amounts of  $\text{Ca}^{2+}$  until we reached the concentration found in DMEM (2 mM) (Fig. 4A). As anticipated, *iT3SS* transcription decreased when the  $\text{Ca}^{2+}$  content of RPMI 1640 was increased, reaching similar levels in the two media when their  $\text{Ca}^{2+}$  contents were similar. Interestingly, the inhibitory effect of INP0341 was much lower than that of INP1750 at low  $\text{Ca}^{2+}$  but similar at a high  $\text{Ca}^{2+}$  concentration. We then examined the influence of serum on *iT3SS* transcription and on INP inhibitory activity (Fig. 4B). Reducing the fetal calf serum (FCS) concentration in the medium caused an impairment of *iT3SS* transcription, as expected. Again, while both inhibitors were equipotent in media with a low FCS content, the inhibitory effect of INP0341 was less than that of INP1750 when the FCS content was increased. Finally, a previous work demonstrated iron-chelating properties for INP0341, leading to a reduction of its inhibitory effect toward intracellular multiplication of *Chlamydia trachomatis* in media enriched in ferric ions (45). We therefore also examined the effect of medium supplementation by ferric ions (over the range used in the *Chlamydia* model) on the inhibition of *iT3SS* transcription by INP0341 or INP1750, but we did not find evidence of any significant effect of the  $\text{Fe}^{3+}$  concentration on the inhibitory activity of INPs (see Fig. S6 in the supplemental material).

In parallel, using CHA and PA103, we examined the effects of INPs on mRNA levels for genes encoding the *iT3SS* effectors (*exoS* [CHA], *exoU* [PA103], and *exoT* [both strains]) and the translocation apparatus (*popB*, *popD*, and *pcrV*). Bacteria were incubated for 3 h (to reach an optical density at 620 nm [ $\text{OD}_{620}$ ] of 0.8) in the presence of

#### FIG 2 Legend (Continued)

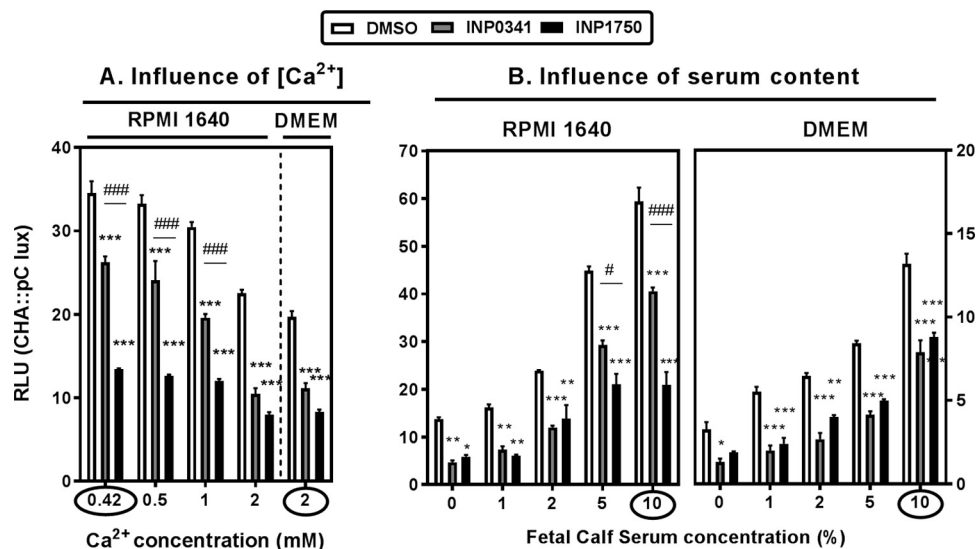
the release of LDH in the culture medium after 5 h (THP-1) or 7 h (A549) of incubation with CHA (10 bacteria/cell) in the presence of increasing concentrations of INP0341 or INP1750. Values are expressed in percentage of inhibition of the cytotoxicity of CHA (as measured in the absence of inhibitors) and are the means  $\pm$  the standard errors of the mean (SEM) of three experiments performed in quadruplicates. Statistical analyses were performed (two-way analysis of variance [ANOVA], Bonferroni posttest; comparison between cells incubated with INP0341 and cells incubated with INP1750 [###,  $P < 0.001$ ; ##,  $P < 0.01$ ]). (B) Cytotoxicity induced by different *P. aeruginosa* strains in THP-1 monocytes (left) or A549 cells (right) after, respectively, 5 and 7 h of incubation in the presence of 0.8% DMSO (vehicle) or 80  $\mu$ M INP0341 or INP1750. Values are expressed as the percentage of LDH release and are means  $\pm$  the SEM of three experiments performed in quadruplicates. Statistical analyses (two-way ANOVA with Bonferroni posttest comparing each of the inhibitors with cells incubated with 0.8% DMSO [\*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ] and comparing the effect of INP0341 and of INP1750 on each strain [#,  $P < 0.05$ ]). (C) IL-1 $\beta$  secretion in the culture medium of THP-1 monocytes preincubated during 4 h in the presence of LPS (100 ng/ml), centrifuged, resuspended in fresh medium, and incubated for 5 h with different bacterial strains (10 bacteria/cell) or nigericin (0.02  $\mu$ M) in the presence of 0.8% DMSO (vehicle) or 80  $\mu$ M INP0341 or INP1750. Values are means  $\pm$  the SEM of two experiments performed in duplicates. Statistical analyses were performed (two-way ANOVA with Bonferroni posttest comparing each of the inhibitors with cells incubated with DMSO [\*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ]; multiple *t* test comparing the effect of INP0341 and of INP1750 on each strain [#,  $P < 0.05$ ]).



**FIG 3** Influence of INP0341 and INP1750 on *iT3SS* transcription. (Top panels) *iT3SS* transcriptional activation of the *exsCEBA* operon, followed by measuring the bioluminescence signal of the reporter strain CHA pC::lux over time, under control conditions (0.8% DMSO) or in the presence of 80 µM INP0341 or INP1750. Bacteria (10/cell) were cultured in the presence of THP-1 monocytes in RPMI 1640 medium (left) or of A549 epithelial cells in DMEM (right). Bioluminescence is expressed in arbitrary units (RLU), which correspond to 10<sup>-4</sup>-fold the actual readings. The dotted lines indicate the time selected in the bottom panels. (Bottom panels) Inhibition of the transcriptional activation of *exsCEBA* operon by increasing concentrations of INP0341 or INP1750, as measured after 5 h of incubation of CHA pC::lux (10 bacteria/cell) in the presence of THP-1 monocytes (left) or after 7 h of incubation in the presence of A549 cells (right). Values are expressed in percentage of inhibition of the bioluminescence signal recorded in the absence of inhibitors. All values are means ± the SEM of two or three experiments. Statistical analyses were performed (two-way ANOVA, Bonferroni posttest; comparison between cells incubated with INP0341 and cells incubated with INP1750 [###, *P* < 0.001; ##, *P* < 0.01]).

80 µM INP0341 or INP1750, or their vehicle, in RPMI 1640 or in DMEM supplemented with 10% FCS before mRNA isolation (Fig. 5A and B) or in Ca<sup>2+</sup>-depleted broth (see Fig. S7 in the supplemental material). Both compounds inhibited the expression of all tested genes. However, INP0341 was less effective in RPMI 1640 than in DMEM; it was also less effective than INP1750 in Ca<sup>2+</sup>-depleted broth. In contrast, INPs did not affect the expression of other genes such as *lasR* and *rhIR* (quorum-sensing regulators) in CHA (Fig. 5A) or the pyocyanin secretion (dependent on quorum sensing) in CHA (see Fig. S8 in the supplemental material), indicating that INPs are not general inhibitors of transcription. Importantly, Phe-Arg-β-naphthylamide (PAβN), a nonspecific efflux pump inhibitor (46), did not modify the inhibitory effects of INPs on gene transcription, suggesting that they are not substrates of the *P. aeruginosa* efflux pumps (data not shown).

The effects of INPs on *iT3SS* gene transcription were also examined using PA103Δ*exsE* and PA103Δ*exsD* strains, which constitutively express the *iT3SS* to high levels (see Fig. 5C for data with PA103Δ*exsE*; comparable results were obtained with PA103Δ*exsD*). Although INP0341 inhibited 30 to 40% of the expression of *iT3SS* genes, INP1750 was ineffective against this strain. These results suggest that INP0341 inhibits *iT3SS* gene



**FIG 4** Influence of culture conditions on iT3SS transcriptional activation and its inhibition by INP0341 and INP1750. The iT3SS transcriptional activation of the *exsCEBA* operon was monitored by measuring the bioluminescence signal of the reporter strain CHA pC::lux after 9 h of incubation in different media and in the presence of 0.8% DMSO (vehicle) or of 80  $\mu$ M INP0341 or INP1750. (A) RPMI 1640 medium containing 10% FCS and supplemented with increasing concentrations of Ca<sup>2+</sup> so as to reach the concentration present in DMEM (circled values correspond to the concentrations present in each medium). (B) RPMI 1640 or DMEM supplemented with increasing amount of FCS (circled values correspond to the concentrations used in the routine). Statistical analyses were performed (two-way ANOVA with Bonferroni posttest comparing each of the inhibitors with cells incubated with DMSO [\*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ]; multiple  $t$  test comparing the effect of INP0341 and of INP1750 on each strain [###,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; #,  $P < 0.05$ ]).

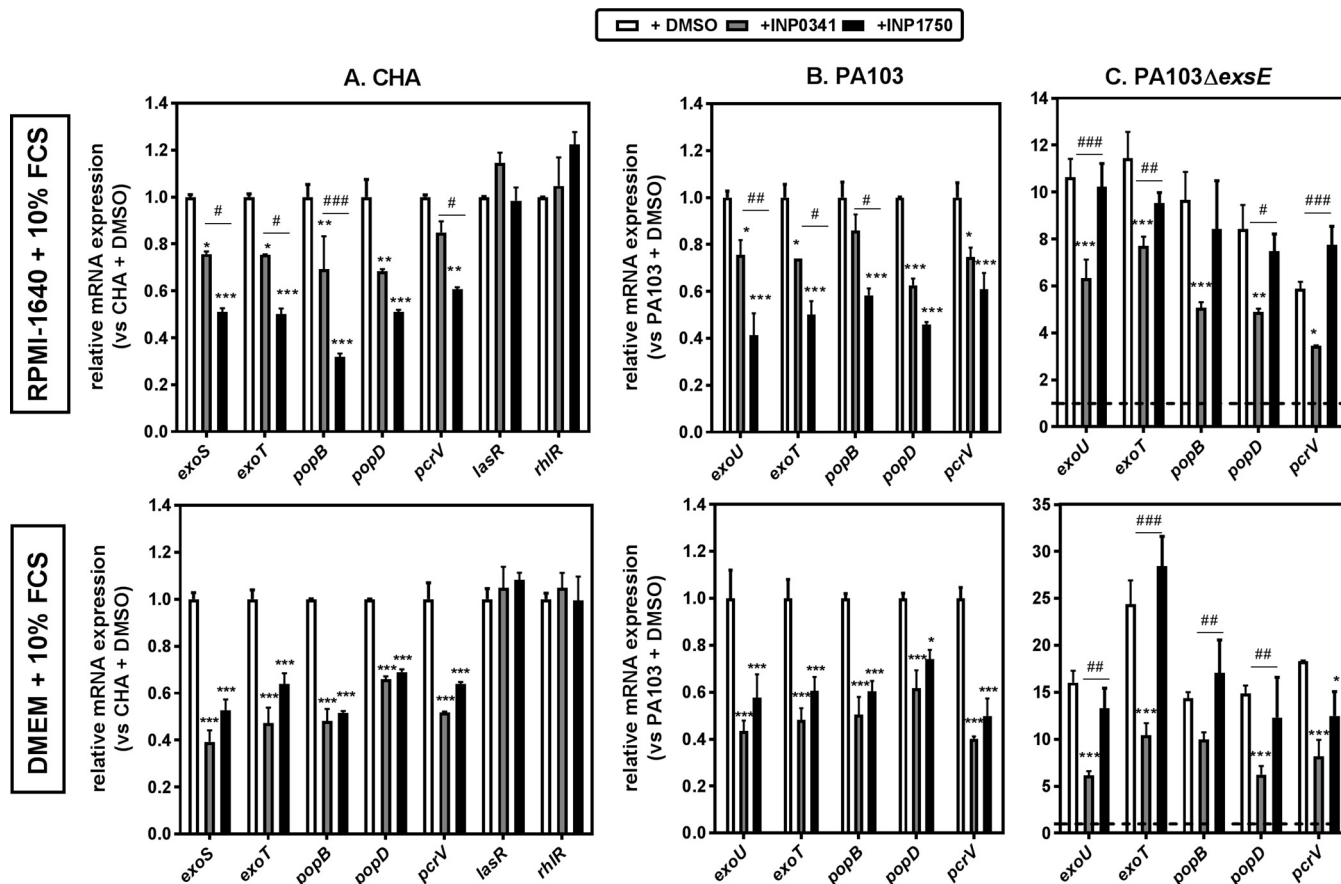
expression, whereas INP1750 most probably acts indirectly by perturbing the *ExsCEBA* regulatory cascade (35).

**Influence of INP0341 and INP1750 on iT3SS secretory activity and bacterial motility.** In a next step, we examined the influence of INPs on the secretion of the ExoS toxin by the iT3SS. To this effect, CHA was incubated for 3 h (to reach an OD<sub>620</sub> of 0.8) in Ca<sup>2+</sup>-depleted broth or in eukaryotic cell culture media (RPMI 1640 plus 10% FCS or DMEM plus 10% FCS) in the presence of 80  $\mu$ M INPs or their vehicle. ExoS was then detected by Western blot in the culture supernatant and in the bacterial lysate (Fig. 6). INP0341 reduced by ~40% the total amount of toxin detected (lysate plus supernatant), suggesting an effect on toxin expression. At the same time, INP0341 also caused a marked decrease in the amount of ExoS detected in the supernatant, possibly due to a lower expression of the machinery responsible for its secretion. INP1750 reduced by ~80% the amount of ExoS detected in the supernatant, with a commensurate increase in the amount detected in the lysate so that the total amount of toxin detected was not modified. This rather suggests an inhibition of the toxin secretion.

The assembly of the bacterial flagellum is also dependent upon a T3SS (fT3SS) distinct from the iT3SS (28). We therefore examined the effect of INP0341 and of INP1750 or their vehicle on bacterial flagellar motility. Bacteria were grown in the presence of 80  $\mu$ M INPs in Ca<sup>2+</sup>-depleted broth and then placed in the center of a 0.5% agar plate and allowed to grow overnight (Fig. 7A). INP1750 reduced the swarming capacity of CHA and its iT3SS-negative mutant CHA $\Delta$ exsA by ~40 to 50%, but INP0341 had only a minimal effect (~10% inhibition for both strains). Comparable results were obtained when examining swimming motility using 0.3% agar, as well as for bacteria precultivated in eukaryotic cell culture media (~40% inhibition for INP1750 versus ~1% inhibition for INP0341). The vehicle had no effect. The concentration dependency of the inhibitory effect of INP1750 on swimming motility is demonstrated in the lower panel for bacteria preincubated with the inhibitor in eukaryotic cell culture media.

Taken together, these data showed that, in contrast to INP0341 that inhibits only iT3SS in our experimental setting, INP1750 also acts upon the fT3SS. We therefore

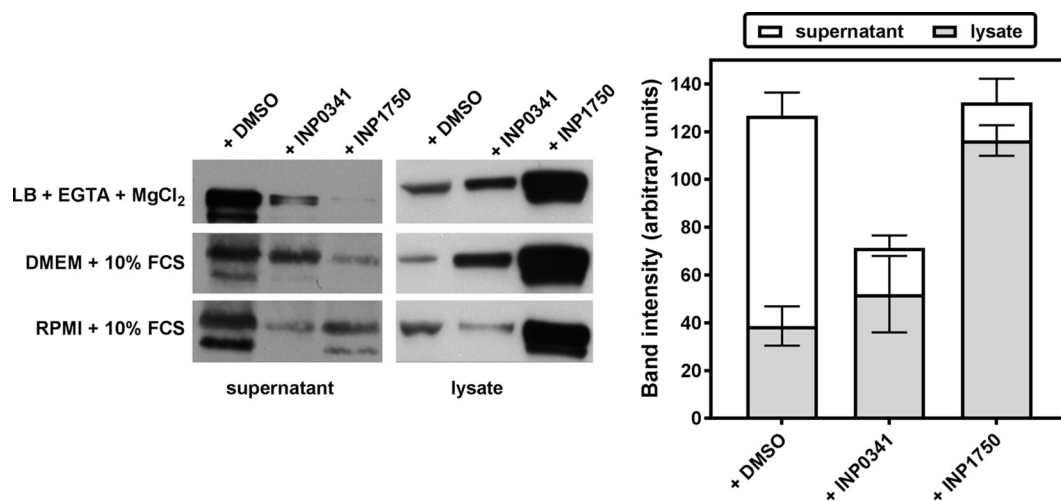




**FIG 5** Influence of INP0341 and INP1750 on medium-dependent *iT3SS* mRNA expression levels. The influence of INP0341 and INP1750 on the expression levels of mRNA encoding *iT3SS* toxins (*exoS* and *exoT*), the *iT3SS* translocation apparatus (*popB*, *popD*, and *pcrV*), and the quorum sensing (QS) transcriptional activator (*lasR* and *rhlR*) was determined by real-time PCR. CHA, PA103, and PA103Δ*exsE* were grown from an OD<sub>620</sub> of 0.1 to an OD<sub>620</sub> of 0.8 with constant shaking in the presence of the 0.8% DMSO (vehicle) or 80 μM INP0341 or INP1750, in eukaryotic cell culture medium (RPMI 1640 plus 10% FCS [top] or DMEM plus 10% FCS [bottom]). The results are expressed in mRNA relative expression compared to CHA (A) or PA103 (B and C) grown in the presence of DMSO. Dotted lines indicate the basal expression levels in PA103 in panel C. Values are means ± the SEM of two or three experiments performed in duplicates. Statistical analyses were performed (two-way ANOVA with Bonferroni posttest comparing each of the inhibitor with cells incubated with DMSO [\*\*\*, *P* < 0.001; \*\*, *P* < 0.01; \*, *P* < 0.05] and comparing the effect of INP0341 and of INP1750 on each mRNA [###, *P* < 0.001; ##, *P* < 0.01; #, *P* < 0.05]).

wondered whether INP1750 could not act by inhibiting the ATPase activity of both T3SSs (that share a high degree of homology (47% identity between PscN [ATPase of *iT3SS*] and Flil [ATPase of *fT3SS*] based on a BLAST sequence alignment [28]). Since these enzymes had not been purified, we used the highly homologous *iT3SS* ATPase from *Y. pseudotuberculosis* (YscN) that shares 57 and 47% identity with *P. aeruginosa* PscN and Flil, respectively (BLAST sequence alignment). As shown in Fig. 7B, INP1750 caused a concentration-dependent inhibition of YscN ATPase activity, whereas INP0341 did not impair the enzymatic activity over the whole range of concentrations tested.

**Influence of INP0341 and INP1750 on cytotoxicity of *P. aeruginosa* clinical isolates.** In order to better delineate the clinical potential of INP0341 and INP1750, we examined their effect on the cytotoxicity induced by clinical isolates collected from patients suffering from acute infections and expressing the *iT3SS* to variable levels (38). These isolates also showed resistance to one or several antibiotic classes (see Table S1 in the supplemental material). THP-1 monocytes or A549 epithelial cells were incubated in the presence of each of these isolates for 5 or 7 h, respectively, after which the cytotoxicity was evaluated by measuring the release of lactate dehydrogenase in the culture medium (Fig. 8). As previously observed, strains expressing ExoU were more cytotoxic than those expressing ExoS (38). In THP-1 monocytes, the cytotoxicity of ExoU-positive strains was not affected by INP0341 but was reduced by 23% by INP1750. The cytotoxicity of ExoS-positive strains was reduced by 33 and 51% by INP0341 and



**FIG 6** Influence of INP0341 and INP1750 on ExoS secretion. CHA was grown from an  $OD_{620}$  of 0.1 to an  $OD_{620}$  of 0.8 with constant shaking in the presence of 0.8% DMSO (vehicle) or 80  $\mu$ M INP0341 or INP1750 in low-calcium medium (LB broth, 5 mM EGTA, 20 mM  $MgCl_2$ ) or in eukaryotic cell culture medium (RPMI 1640 plus 10% FCS or DMEM plus 10% FCS). ExoS was detected by Western blotting after separation by SDS-PAGE of 13  $\mu$ l (supernatants) or 5  $\mu$ g (lysates) of proteins. The band intensity was quantified by using ImageJ software.

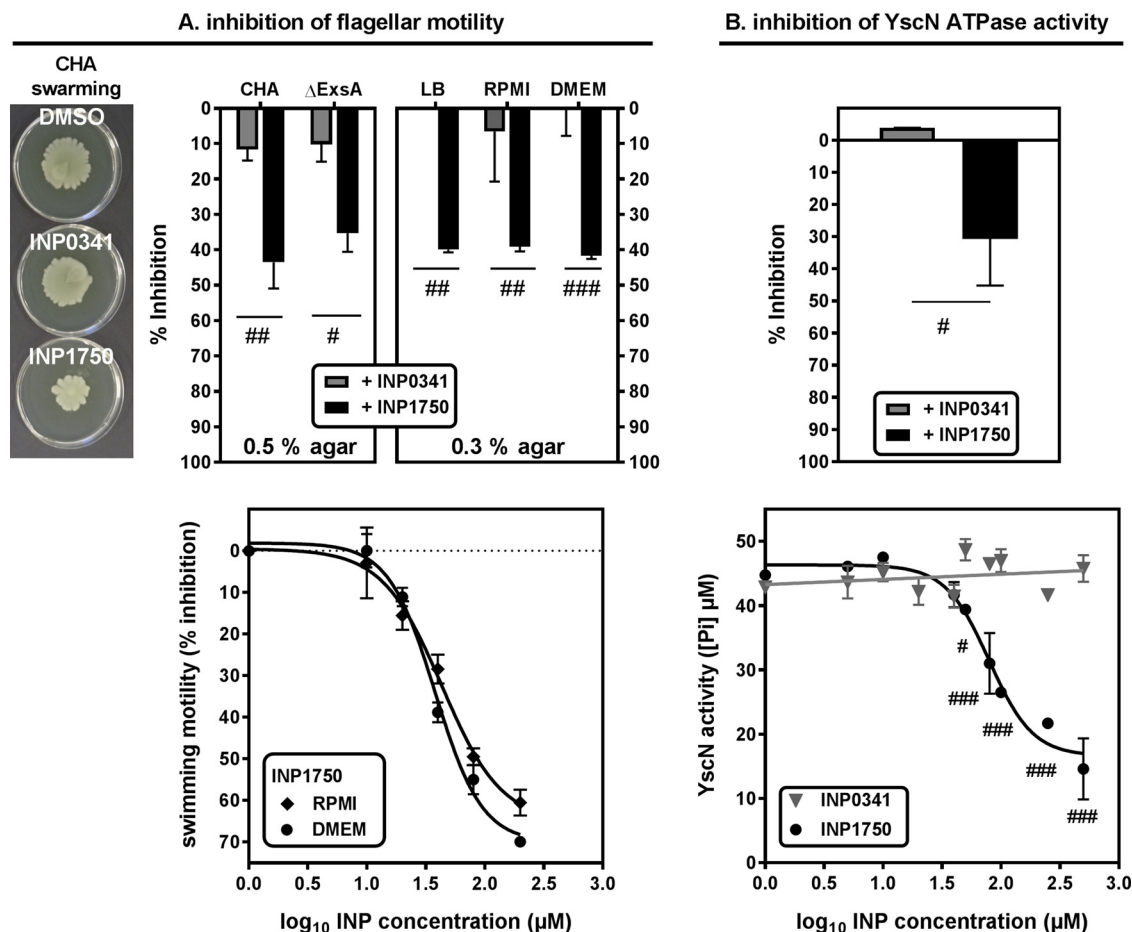
INP1750, respectively. In A549 epithelial cells, INP0341 and INP1750 reduced cytotoxicity by 18 to 20% for isolates expressing ExoU and by 54 and 35%, respectively, for isolates expressing ExoS (the difference in control values was statistically significant only for isolates expressing high levels of ExoS).

## DISCUSSION

In this study, we compare the antivirulence properties of two molecules belonging to distinct chemical families (the salicylidene acylhydrazide INP0341 and the hydroxyquinoline INP1750) toward *P. aeruginosa*, which were originally described as inhibitors of *iT3SS* transcription in *Y. pseudotuberculosis*, *Shigella*, *Salmonella*, and *Chlamydia* spp. (31–33).

Both compounds protect eukaryotic cells from the toxic effects mediated by the *iT3SS* itself or by the toxins, which results in the release of mature IL-1 $\beta$  following inflammasome activation, the prevention of bacteria internalization, and the induction of cell necrosis. However, despite similarities in these protective effects, the two INPs differ in their direct effects on bacteria, suggesting that they act upon distinct pharmacological targets.

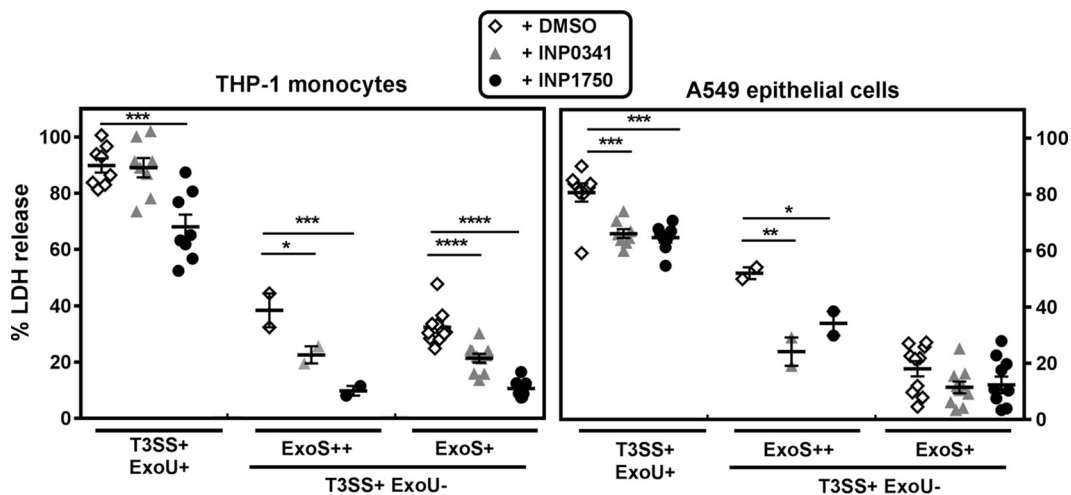
For the salicylidene acylhydrazide INP0341, we present two convergent pieces of evidence suggesting that it targets *iT3SS* gene transcription. First, INP0341 reduces the total amount of ExoS toxin detected by Western blot in treated cultures of CHA, a finding consistent with a reduction in its synthesis following impaired gene transcription. Second, INP0341 reduces *iT3SS* gene expression and *iT3SS*-mediated toxicity as efficiently in wild-type strains and in mutants that constitutively express *iT3SS*, indicating a direct effect on gene transcription. However, INP0341 does not affect the *iT3SS* (flagellum), as opposed to other salicylidene acylhydrazides that were shown to inhibit the flagellar motility of *Salmonella enterica* serovar Typhimurium (33). Although this difference may result from variations in the protocol used to assess flagellar motility (preincubation with INPs before inoculation in the agar here versus the addition of INPs in the agar in reference 33), INP0341 is nevertheless distinct from INP1750 in this context. The molecular mechanism by which inhibition of transcription by INP0341 takes place remains to be established and could be indirect. An interaction with specific proteins (namely, WrbA [NADP(H):quinone reductase], Tpx [thiol peroxidase], and FolX [epimerase]) has been demonstrated for other salicylidene acylhydrazides in *Escherichia coli* and *Yersinia pseudotuberculosis* (47, 48) and proposed to indirectly interfere with *iT3SS* regulation by altering cellular metabolism. Homologs of these enzymes are



**FIG 7** Influence of INP0341 and INP1750 on bacterial motility and YscN-ATPase activity. (A) Effect of INP0341 and INP1750 on flagellar motility. (Top) Swarming (left) and swimming (right) motility. Bacteria were grown from an OD<sub>620</sub> of 0.1 to an OD<sub>620</sub> of 0.8 with constant shaking in the presence of 0.8% DMSO (vehicle) or 80 μM INP0341 or INP1750; then, 3-μl portions of these cultures were placed in the center of agar LB plates and grown overnight at 37°C. The area covered by bacteria was evaluated using QuantityOne software (Bio-Rad). Swarming was evaluated using 0.5% agar for CHA (illustrated in the pictures) and CHAΔ*exsA* strains that were pre-cultivated with INPs in low-calcium medium (LB broth, 5 mM EGTA, 20 mM MgCl<sub>2</sub>) and swimming using 0.3% agar for CHA pre-cultivated with INPs in low-calcium medium or in RPMI 1640 or DMEM added by 10% serum (right). (Bottom) Concentration effect relationship for swimming, with bacteria grown with increasing concentrations of INP1750 in RPMI 1640 or DMEM added by 10% serum, as described above. Values are means ± the SEM of two independent experiments performed in duplicate. Statistical analyses were performed (#, *P* < 0.05; ##, *P* < 0.01; ###, *P* < 0.001). A Student *t* test was used to compare values measured in the presence of INP0341 or in the presence of INP1750. (B) Effect of INP0341 and INP1750 on purified YscN T3SS ATPase. His-YscN (25 μg/ml) was incubated with INP0341 or INP1750 for 1 h; ATP (4 mM) was then added to the samples for 30 min, and the ATPase activity was measured as the amount of free phosphate liberated. Experiments were performed in duplicates. (Top) Percentage of inhibition of the enzymatic activity in the presence of 80 μM concentrations of each INP. (Bottom) Residual activity as a function of INP concentrations. Statistical analyses were performed (#, *P* < 0.05; ##, *P* < 0.01; ###, *P* < 0.001). A Student *t* test was used to compare values measured in the presence of INP0341 or in the presence of INP1750.

present in *P. aeruginosa* but could not be examined as potential targets for INP0341 in the present study.

For the hydroxyquinoline INP1750, our results point to a mechanism of action similar to what we previously described for INP1855, another hydroxyquinoline, consisting in an inhibition of a core component of T3SS sharing homology between iT3SS and fT3SS (35). Like INP1855, INP1750 indeed causes a drastic reduction of ExoS secretion (without reducing the total amount of toxin produced by the bacteria) and of flagellar motility, together with an inhibition of the activity of an enzyme homologous to the ATPases present in both T3SSs. As opposed to INP0341, it does not inhibit iT3SS transcription in constitutive mutants of PA103, while still decreasing their cytotoxicity. In the case of INP1750, inhibition of iT3SS transcription is probably not a primary effect but rather an indirect consequence of the inhibition of the secretory activity of iT3SS. In addition to



**FIG 8** Influence of INP0341 and INP1750 on the cytotoxicity of *P. aeruginosa* clinical isolates. The percentage of LDH release from THP-1 monocytes (left) or A549 cells (right) exposed for 5 h (left) or 7 h (right) to clinical isolates (10 bacteria/cell) in the presence of 0.8% DMSO (control; open symbol) or 80  $\mu$ M INP0341 or INP1750 was determined. Strains were grouped according to the expression of ExoU toxin (T3SS+ ExoU+ versus T3SS+ ExoU-) or of the ExoS toxin (ExoS++, level of expression higher than that detected in CHA; ExoS+, level of expression lower than that detected in CHA). Values are means  $\pm$  the SEM of three independent experiments performed in quadruplicate (LDH). Statistical analyses were performed (\*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$  [two-way ANOVA, Bonferroni posttest]) comparing control conditions with INP0341 or INP1750.

ExoS, iT3SS indeed also secretes other proteins, including FlhC and ExsE (the negative regulator of *iT3SS* gene expression). We previously showed that the hydroxyquinoline INP1855 impairs the secretion of these three proteins (ExoS, flagellin, and ExsE), leading us to conclude that *iT3SS* transcription by this compound is indirectly inhibited as a consequence of the increase in ExsE content inside the bacteria (35).

In addition to their mode of action, INP0341 and INP1750 also differ in their intrinsic activity, INP0341 being less potent than INP1750 through all the parameters investigated in this study. This difference could be due to the existence of distinct targets, but also to the differential influence exerted by the composition of the culture medium on their activity. We observe indeed that INP0341 is as active as INP1750 in DMEM but is less active in RPMI 1640. RPMI 1640 has a lower  $Ca^{2+}$  content than DMEM, which favors *iT3SS* expression (21, 23–25), and may consequently counteract the INP0341 activity on gene transcription. However, serum, which also increases *iT3SS* expression, affects to the same extent the activity of both INP0341 and INP1750 in  $Ca^{2+}$ -rich DMEM. This therefore suggests another role of  $Ca^{2+}$  concentration, independent of its influence on *iT3SS* transcription level, in the modulation of INP0341 activity. A previous work with *C. trachomatis* failed to show an influence of  $Ca^{2+}$  concentration on INP0341 activity (45), but  $Ca^{2+}$  supplementation was performed in a medium as rich in  $Ca^{2+}$  as DMEM, in which INP0341 activity was already maximal in our setting. Conversely, that study also showed that the activity of INP0341 on *C. trachomatis* was impaired in  $Fe^{3+}$ -enriched medium due to the iron-chelating properties of salicylidene hydrazides (45, 49). We did not observe any modification in the antipseudomonal activity of INP0341 upon medium supplementation in  $Fe^{3+}$ , and we postulate therefore that its mode of action is probably different from that described against *C. trachomatis*. In addition to its effect on *iT3SS* transcription, serum also increases the outer membrane permeability of *P. aeruginosa* (50), which could modulate the activity of *iT3SS*, as well as its inhibition by INP0341, but this was not investigated here.

Moving now to INP1750, we see that its activity is only minimally influenced by the medium composition (RPMI 1640 or DMEM) whatever their  $Ca^{2+}$  or serum content. INP1750 is also equipotent to INP1855 (35) when comparing their effects on bacteria (*iT3SS* secretory activity and transcription, flagellar motility, and inhibition of ATPase activity) and their consequences for the host cells (protection against necrosis and inflammasome activation).

Our work suffers from two main limitations. First, we did not identify at this stage the molecular target of these compounds, which would require additional experiments, including using global approaches such as transcriptomics or proteomics. The experimental approaches we set up could nevertheless represent a useful panel of techniques for the high-throughput screening of T3SS inhibitors in order to get a first insight of their main mechanism of action and also to screen larger series of compounds in order to discover more active derivatives based on refined structure-activity relationships. Applied to hydroxyquinolines and salicylidene acylhydrazides, these techniques allowed us to position them as a potentially useful addition to our future therapeutic armamentarium, since both show activity against clinical isolates, disregarding their resistance profile to currently used antipseudomonal antibiotics. Compared to previously described inhibitors of iT3SS in *P. aeruginosa* (see reference 22 for a review), they may offer a broader spectrum of activity than (i) compounds blocking toxins only (51–53), the activity of which will probably be restricted to toxin-producing strains, and (ii) antibodies for which activity can be variable due to the polymorphism of the targeted antigens (54). The present data, together with the results obtained previously with INP1855 (35), strongly suggest an advantage for hydroxyquinolines over salicylidene acylhydrazides. Hydroxyquinolines, indeed, show better effects on both iT3SS and the fT3SS, two key determinants in the pathogenicity of *P. aeruginosa* (3, 11, 12, 27, 55).

Second, we have not compared the activity of the two compounds *in vivo*. However, studies with other compounds in these families and/or with other bacterial species have demonstrated that they could be active *in vivo* (35, 36). Based on the in-depth pharmacological comparison performed here, future work is therefore warranted in order to characterize both their pharmacokinetics and their tolerance. This will allow us to define the best experimental conditions under which their efficacy could be studied in animal models of acute pseudomonal infection.

## MATERIALS AND METHODS

**Bacterial strains, growth conditions, and susceptibility testing.** The strains and plasmids used here were described in a previous publication (35). PA103 is a cytotoxic strain expressing the phospholipase ExoU (56). CHA is a clinical strain isolated from a cystic fibrosis patient and expresses the ExoS/T toxins to a high level (57). The bioluminescent reporter strain CHA (pC::lux) was constructed by transcriptional fusion of *Photobacterium luminescens lux* operon (*luxCDABE*) to the promoter of the iT3SS regulator operon *exsCEBA* (25). A series of strains derived from PA103 and CHA with deletions in the iT3SS regulon, the genes encoding iT3SS effector toxins or proteins from the translocon apparatus were also included in the study, together with 20 clinical isolates collected from acute infections in four Belgian hospitals (38). Unless stated otherwise, all strains were routinely grown overnight in Luria-Bertani (LB) broth (Becton Dickinson, Franklin Lakes, NJ) at 37°C with constant shaking (130 rpm) and under aerobic conditions. MICs were determined by geometric microdilution in cation-adjusted Mueller-Hinton broth (BD Diagnostics). Susceptibility was assessed according to the current interpretive criteria of both the European Committee on Antimicrobial Susceptibility Testing and the Clinical and Laboratory Standards Institute.

**Cells and culture.** Human THP-1 cells (ATCC TIB-202) are a myelomonocytic cell line showing moderate phagocytic activity and low cell defense mechanisms (58). They were grown in suspension in RPMI 1640 supplemented with 10% fetal calf serum (FCS; Gibco/Life Technologies Corporation, Paisley, UK). Human alveolar epithelial A549 cells (ATCC CCL-185) are adherent cells derived from lung carcinoma (59). In contrast to THP-1 monocytes, these cells do not express NLRC4 (60). They were cultured in DMEM supplemented with 10% FCS and seeded in culture plates for 48 h prior being used for experiments. Cells were counted using a Burkert hemocytometer to adjust their concentration prior infection.

**T3SS inhibitors.** INP0341 and INP1750 were kindly provided by Creative Antibiotics Sweden AB (Umeå, Sweden). Stock solutions were prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at room temperature in the dark for less than 1 month according to the manufacturer's instructions.

**Cytotoxicity assay.** The cytotoxicity of INP0341 or INP1750 and of *P. aeruginosa* strains was determined by measuring the release of the cytoplasmic enzyme LDH in the culture medium, which occurs upon cell necrosis (61). Cells were seeded into 96-well plates (at a density of  $2.5 \times 10^5$  cells/ml for phagocytic cells and of  $8 \times 10^4$  cells/ml for epithelial cells) and incubated with INP0341 or INP1750 or/and with *P. aeruginosa* strains (10 bacteria/cell) for 5 h (THP-1 monocytes) or 7 h (A549 epithelial cells). The amount of LDH released was determined by using a cytotoxicity detection kit (Plus; Roche Diagnostics, Basel, Switzerland).

**Internalization of *P. aeruginosa* in THP-1 cells.** Bacteria internalization was evaluated using a previously described protocol (aminoglycoside protection assay [62]) with minor adaptations. Overnight

bacterial cultures were pretreated during 3 h with INP0341 or INP1750 in (0.8%) DMSO or with the vehicle, resuspended in eukaryotic cell medium with 10% human serum in the presence of INPs or DMSO, and incubated for 1 h at 37°C to allow for opsonization. Bacteria were then added to THP-1 monocytes (10 bacteria/cell;  $7.5 \times 10^5$  cells/ml), again in the presence of INPs or DMSO. Infected cells were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 2 h to allow phagocytosis, washed in phosphate-buffered saline (PBS), and then exposed to tobramycin at 200 mg/liter for 1 h to kill extracellular bacteria. After three washes with PBS, the cells were lysed in 1 ml of sterile water, and aliquots were plated on tryptic soy agar plates after suitable dilution for the determination of CFU by colony counting after 24 h of incubation at 37°C. The results were normalized per mg of cell protein, as determined by Lowry's assay using a commercially available detection kit (Bio-Rad DC protein assay; Bio-Rad Laboratories, Hercules, CA).

**Cytokine release assay.** THP-1 cells ( $2.5 \times 10^5$  cells/ml) were preincubated during 4 h with lipopolysaccharide (LPS; Sigma-Aldrich) at 100 ng/ml in order to stimulate the production of pro-IL-1 $\beta$ . The cells were then incubated for 5 h with INPs or DMSO alone or combined with (i) *P. aeruginosa* strains (10 bacteria/cell) or (ii) 0.02  $\mu$ M nigericin, after which the cytokines released into the supernatant were measured using commercially available enzyme-linked immunosorbent assay kits (IL-1 $\beta$  [R&D Systems, Minneapolis, MN]; TNF- $\alpha$  [BD Biosciences, San Jose, CA]).

***P. aeruginosa* growth curves.** Overnight cultures were pelleted and resuspended in LB broth at an initial OD<sub>620</sub> of 0.1; the increase in OD<sub>620</sub> was then monitored over time during incubation at 37°C with aeration and constant shaking (300 rpm) in the presence of INP0341 and INP1750 in 2.5% DMSO or in 2.5% DMSO only (vehicle). Since some experiments were performed in the presence of eukaryotic cells, the growth rates were also measured in RPMI 1640 or DMEM supplemented by 10% FCS. No differences were observed compared to what was seen in LB broth (see Fig. S2 in the supplemental material).

**Reporter genes assay.** An overnight culture of CHA (pC::lux) was centrifuged and resuspended in eukaryotic cell culture medium (supplemented by 10% of FCS) and added to THP-1 monocytes or A549 epithelial cells ( $5 \times 10^5$  cells/ml; 10 bacteria/cell) in the presence of increasing concentrations of INPs in DMSO ( $\leq 0.8\%$  DMSO) or of DMSO only. Cultures were incubated without shaking at 37°C in a 5% CO<sub>2</sub> atmosphere. Luminescence was measured in relative light units (RLU) over time using a SpectraMax spectrofluorometer (Molecular Devices LLC, Sunnyvale, CA). Concentration-response curves were also obtained after 5 h (THP-1) or 7 h (A549) of incubation (see reference 35 for a justification of these incubation times).

**Real-time PCR.** Overnight cultures were resuspended in LB broth containing 5 mM EGTA and 20 mM MgCl<sub>2</sub> or in eukaryotic cell medium (RPMI 1640 or DMEM) supplemented by 10% of FCS and grown from an OD<sub>620</sub> of 0.1 to an OD<sub>620</sub> of 0.8 with aeration and constant shaking (300 rpm) in the presence of INPs in 0.8% DMSO or 0.8% DMSO only. Total RNA was extracted by using an RNeasy minikit (Stratag Molecular GmbH, Berlin, Germany) and reverse transcribed using a Transcriptor first-strand kit (Roche Diagnostics) according to the manufacturer's instructions. RNA purity was checked for the absence of contaminating DNA prior to reverse transcription by PCR amplification of a fragment of the *rpsL* gene. Amplification reactions were performed in the presence of the Sybr green IQ Supermix (Bio-Rad Laboratories), using an iCycler iQ single-color real-time PCR detection system (MyiQreal Time PCR software; Bio-Rad). Relative quantification of the mRNA levels was obtained by the comparative C<sub>T</sub> ( $2^{-\Delta\Delta C_T}$ ) method (63), using *rpsL* as a housekeeping gene. The primers and conditions were as previously described (35, 38).

**Pyocyanin production assay.** Bacteria were grown overnight in 5 ml of LB broth at 37°C with shaking at 130 rpm in the presence of INP0341 or INP1750 in DMSO or of DMSO only. After centrifugation at 4,000 rpm for 15 min, pyocyanin was extracted from the supernatant successively with 2.5 ml of chloroform and 0.2 N HCl and quantified by measuring the absorbance of the solution at 520 nm, based on a calibration curve established with purified pyocyanin (Sigma-Aldrich, St. Louis, MO) (64).

**Detection of ExoS by Western blot.** Overnight cultures were resuspended in LB broth containing 5 mM EGTA and 20 mM MgCl<sub>2</sub> or in eukaryotic cell medium (RPMI 1640 or DMEM) and grown from an OD<sub>620</sub> of 0.1 to an OD<sub>620</sub> of 0.8 with aeration and constant shaking (300 rpm) in the presence of INPs in 0.8% DMSO or of 0.8% DMSO only. Lysates were centrifuged (10 min at 4,000 rpm), and the pellet was collected, washed in ice-cold PBS, resuspended in radioimmunoprecipitation assay buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% sodium deoxycholate, and a cocktail of protease phosphatase inhibitors diluted according to the manufacturer's instructions [Sigma-Aldrich; catalog no. P8340]), and subjected to sonication. The gross debris were then eliminated by centrifugation for 15 min at  $20,800 \times g$ . Supernatants (5 ml) were concentrated by centrifugation (10 min;  $4,200 \times g$ ) through Amicon Ultra 4 centrifugal filters (Millipore; Merck KGaA, Darmstadt, Germany) in order to reduce the volume to 100  $\mu$ l. The protein content of both preparations was measured using a bicinchoninic acid protein assay (Bradford assay; BCA reagents; Pierce, Rockford, IL). This protein content was similar in all samples. Appropriate quantities of proteins (corresponding to the same volume for all samples) were mixed to 4 $\times$  NuPAGE LDS sample buffer and 10 $\times$  NuPAGE reducing agent and then heated for 10 min at 70°C. Samples were loaded on acrylamide gels (NuPAGE 10% Bis-Tris gel; Invitrogen, Carlsbad, CA). The proteins were electrotransferred after migration onto a polyvinylidene difluoride membrane, which was blocked by 1 h of incubation with 5% defatted milk in Tris-buffered saline (20 mM Tris-HCl, 500 mM NaCl [pH 7.5]) containing 0.1% Tween 20. The membranes were then incubated overnight with anti-ExoS antibody (Agrisera AB, Vännäs, Sweden; 1/5,000 dilution) and then with an appropriate horseradish peroxidase-coupled secondary antibody (1/10,000 dilution) for 1 h. Blots were revealed by chemiluminescence (SuperSignal West Pico; Pierce/Thermo Fisher Scientific, Inc., Rockford, IL). The band intensity was quantified using ImageJ software (v1.47; <http://imagej.nih.gov/ij/>).

**Motility assay.** Overnight cultures were resuspended in LB broth or eukaryotic cell medium (RPMI 1640 or DMEM) with DMSO or INP0341 or INP1750 and grown from an OD<sub>620</sub> of 0.1 to an OD<sub>620</sub> of 0.8

with aeration and constant shaking (300 rpm) (65). Portions (3  $\mu$ l) of these cultures were placed at the center of a 0.3% (swimming motility) or 0.5% (swarming motility) LB agar plate. Plates were incubated for 20 h at 37°C, and the dissemination area was evaluated using QuantityOne software (v4.5.0; Bio-Rad Laboratories).

**ATPase purification and enzymatic activity assay.** Because the PscN ATPase of the iT3SS of *P. aeruginosa* has not been fully purified so far (35), we used the highly homologous YscN enzyme from *Yersinia pseudotuberculosis*, for which a vector encoding the His-tagged enzyme was available (57% identity; 72% positive substitutions with PscN by BLAST alignment). The enzyme was purified exactly as previously described (66), and its activity was assessed using a MAK113 kit (Sigma-Aldrich) according to the recommendations of the manufacturer.

**Curve fittings and statistical analyses.** Curve fittings were performed with Prism (v7.02), and statistical analyses were completed using InStat (v3.1), both from GraphPad Prism Software, San Diego, CA.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.02566-16>.

**SUPPLEMENTAL FILE 1**, PDF file, 1.1 MB.

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**Salicylidene acylhydrazides and hydroxyquinolines act as inhibitors of type three secretion systems in *Pseudomonas aeruginosa* by distinct mechanisms.**

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**SUPPLEMENTAL MATERIAL**

**Table S1** : MICs values and susceptibility patterns<sup>a</sup> of the clinical strains of *Pseudomonas aeruginosa*

	Strains	Antibiotics <sup>b</sup>												
		GEN	AMK	TOB	MEM	ATM	TIC	PIP	TZP	CAZ	FEP	CIP	CST	
clinical isolates	T3SS+ ExoU+	13846184	4	16	0.5	<b>16</b>	<b>64</b>	<b>128</b>	16	16	<b>32</b>	<b>16</b>	0.125	1
		9101/2	<b>16</b>	<b>128</b>	<b>256</b>	<b>32</b>	<b>32</b>	<b>256</b>	<b>128</b>	<b>32</b>	8	<b>16</b>	<b>64</b>	1
		14081972	2	4	0.5	<b>16</b>	8	<b>32</b>	8	4	2	4	0.25	1
		14241108	<b>&gt;512</b>	<b>128</b>	<b>128</b>	<b>32</b>	<b>&gt;512</b>	<b>&gt;512</b>	<b>&gt;512</b>	<b>256</b>	<b>256</b>	<b>256</b>	<b>8</b>	1
		2504/6	<b>32</b>	1	<b>32</b>	2	16	<b>128</b>	<b>128</b>	<b>64</b>	<b>16</b>	<b>16</b>	1	1
		24138438	4	4	1	<b>8</b>	16	<b>64</b>	<b>32</b>	32	8	4	0.5	2
		24139146	2	4	0.5	1	16	<b>32</b>	4	8	2	4	0.125	4
		24138943	<b>64</b>	1	<b>32</b>	2	<b>32</b>	<b>128</b>	<b>512</b>	<b>128</b>	<b>16</b>	<b>16</b>	<b>8</b>	2
	T3SS+ ExoU-	NSIH 4603	4	4	1	1	16	<b>32</b>	8	8	4	8	0.125	2
		9101/1	0.5	1	0.125	<b>64</b>	<b>512</b>	<b>512</b>	<b>32</b>	<b>32</b>	8	<b>64</b>	0.5	1
		ZIV889	4	4	0.5	4	16	<b>64</b>	4	8	2	8	0.5	1
		24134699	2	4	1	4	8	<b>32</b>	4	16	2	4	0.125	4
		24140250	4	4	1	0.25	16	<b>32</b>	8	8	2	4	0.125	1
		05/1592	4	8	0.5	<b>32</b>	<b>64</b>	<b>128</b>	<b>32</b>	<b>32</b>	8	<b>16</b>	<b>4</b>	0.25
		15031978	1	4	1	0.5	<b>32</b>	<b>128</b>	16	16	8	8	0.25	1
		24128193	0.5	1	0.25	1	<b>32</b>	<b>64</b>	16	16	8	8	0.5	2
		BG0501/9344	1	2	1	0.5	8	<b>32</b>	8	8	2	2	0.25	0.25
		24138431	1	4	0.5	<b>8</b>	<b>32</b>	<b>64</b>	16	8	4	4	0.5	1
		24137296	4	4	1	0.5	1	<b>32</b>	8	8	2	4	0.5	1
ZKT097	2	4	0.5	0.5	4	16	4	4	2	4	0.125	4		

<sup>a</sup> values in bold characters are above the susceptibility breakpoints of CLSI (black), EUCAST (blue), or both CLSI and EUCAST (red), highlighting resistance

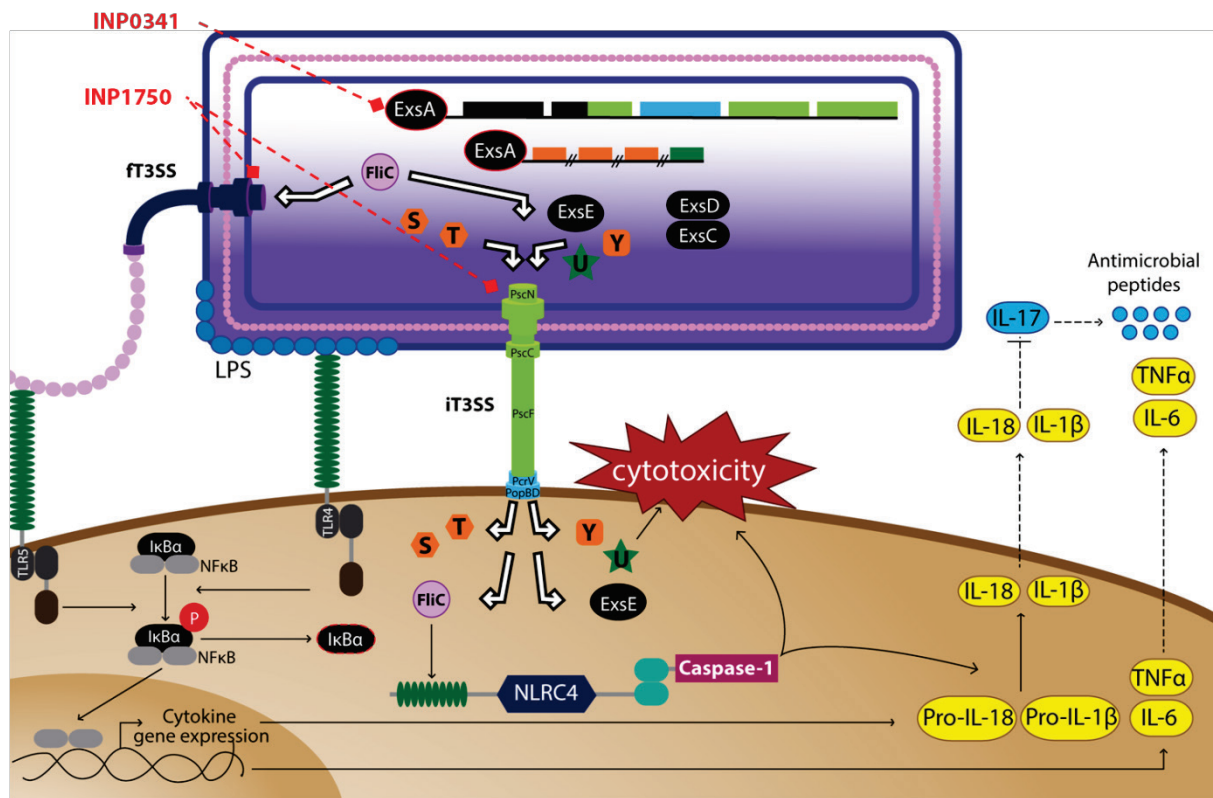
<sup>b</sup> GEN: gentamicin; AMK: amikacin; TOB: tobramycin; MEM: meropenem; ATM: aztreonam; TIC: ticarcillin; PIP: piperacillin; TZP: piperacillin-tazobactam; CAZ: ceftazidime ; FEP : cefepime ; CIP : ciprofloxacin ; CST : colistin

**Figure S1: Pictorial view of the physiopathology of cytotoxicity mediated by iT3SS and fT3SS and of the putative targets of INP0341 and INP1750.**

The bacterium is represented in violet at the top of the figure; the eukaryotic cell (focusing on a cell expressing the NLRC4 inflammasome), in brown on the bottom.

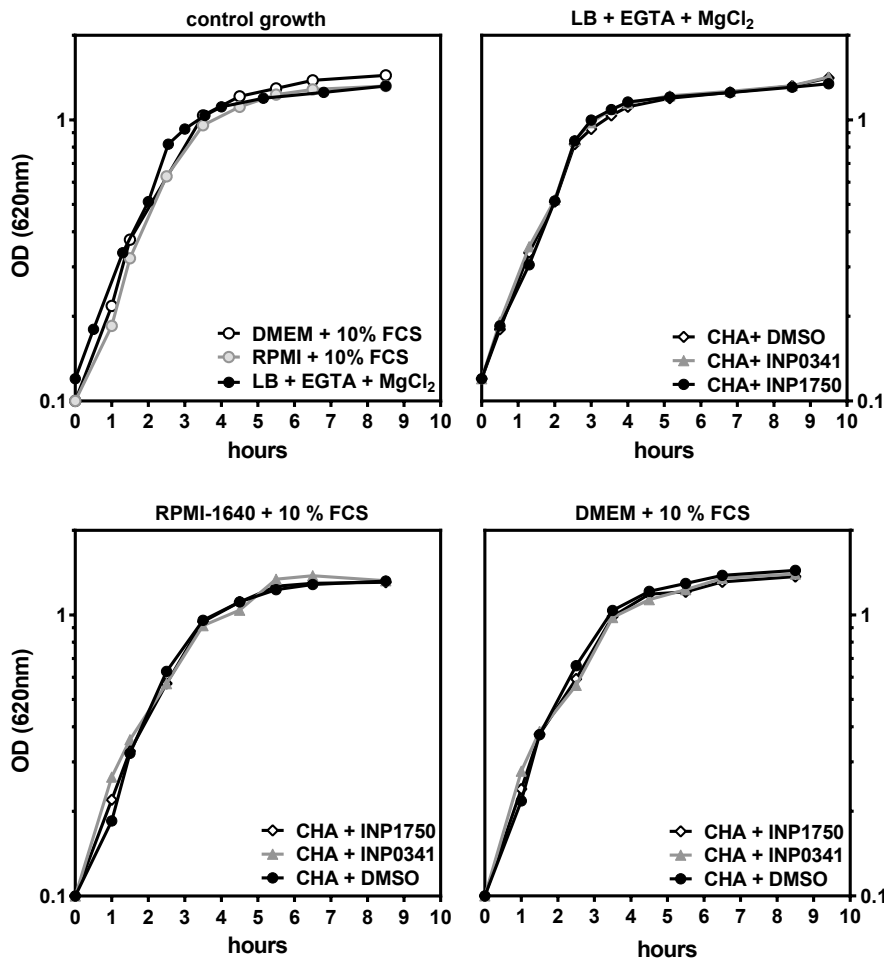
In control conditions (no inhibitor), iT3SS (injectisome) can inject several proteins into the host cell cytosol (see white arrows pointing to secretion/translocation), among which the exoenzymes S, T, Y or U, the negative regulator ExsE, and the FliC protein (monomeric subunit of flagellum). The latter can activate the NLRC4 inflammasome, causing caspase-1 activation. The active enzyme cleaves pro-IL-1 $\beta$  to IL-1 $\beta$  and pro-IL-18 to IL-18, which are released out of the cell and inhibit IL-17 signaling. LPS or fT3SS (flagellum) can activate the NF $\kappa$ B pathway via TLR4 or TLR5 signaling and phosphorylation of I $\kappa$ B $\alpha$ .

This work suggests that INP0341 acts on iT3SS by inhibiting iT3SS transcription via a still unknown mechanism, while INP1750 inhibits a core protein of the basal body of iT3SS and fT3SS, presumably their ATPase. Both molecules inhibit the secretion/translocation of proteins by iT3SS (including FliC), which prevents caspase-1 activation and subsequent IL-1 $\beta$  release. INP1750 also impairs flagellar motility.



Adapted with permission from: Anantharajah A., E. Faure, J. M. Buyck, C. Sundin, T. Lindmark, J. Meccas, T. L. Yahr, P. M. Tulkens, M. P. Mingeot-Leclercq, B. Guery, and R1.5.F. Van Bambeke. 2016. Inhibition of the injectisome and flagellar type III secretion systems by INP1855 impairs *Pseudomonas aeruginosa* pathogenicity and inflammasome activation. *J. Infect. Dis.* 214:1105-1116. PMID: 27412581

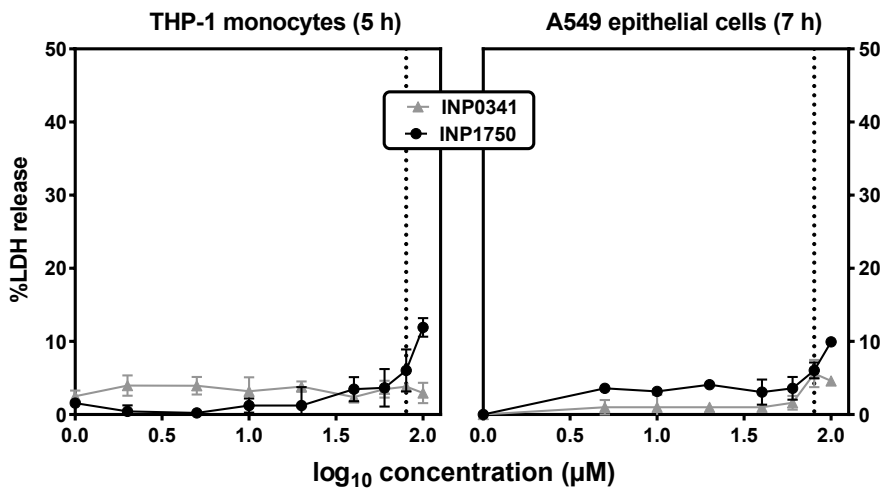
**A. bacteria (CHA) growth curve: influence of medium and INPs**



**Figure S2: Influence of INP0341 and INP1750 on bacterial growth and on eukaryotic cell viability.**

A. Influence of the culture medium and of INP0341 and INP1750 on growth rate of bacteria, as assessed by following over time the optical density at 620 nm of a suspension of CHA in different media and in the absence or in the presence of 250 μM of inhibitors or of 2.5 % DMSO (vehicle).

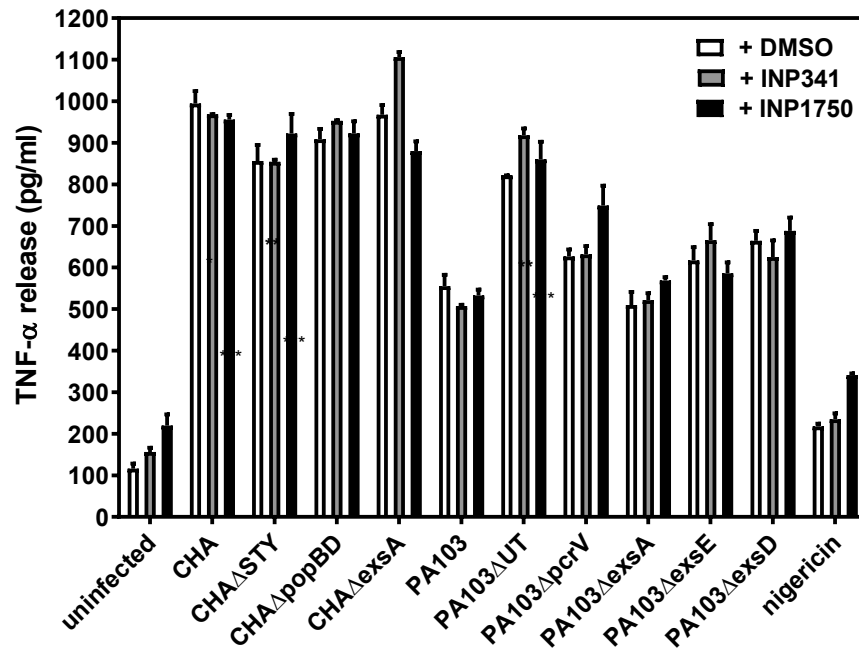
**B. eucaryotic cells viability**



B. Effect of INP0341 and INP1750 on viability of eukaryotic cells, as assessed by measuring the release of lactate dehydrogenase (LDH) in the culture medium after 5 h (THP-1) or 7 h (A549) of incubation with increasing concentrations of inhibitors. The vertical dotted line corresponds to the concentration of 80 μM used in most experiments.

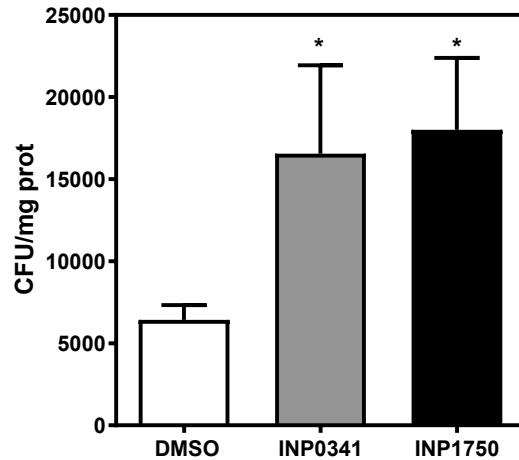
**Figure S3: Influence of INP0341 and INP1750 on TNF- $\alpha$  secretion induced by *P. aeruginosa* strains**

THP-1 monocytes were pre-incubated during 4 h in the presence of LPS (100 ng/mL) and Brefeldin A (10  $\mu$ g/mL), after which cells were centrifuged, washed and incubated during 5 h with different bacterial strains (10 bacteria/cell) or of nigericin (0.02  $\mu$ M) in the presence of 0.8 % DMSO (vehicle) or 80  $\mu$ M INP0341 or INP1750 in 0.8 % DMSO. TNF- $\alpha$  was then measured in the supernatant. All values are the mean  $\pm$  SEM (n=2).



**Figure S4: Influence of INP0341 and INP1750 on CHA phagocytosis by THP-1 cells.**

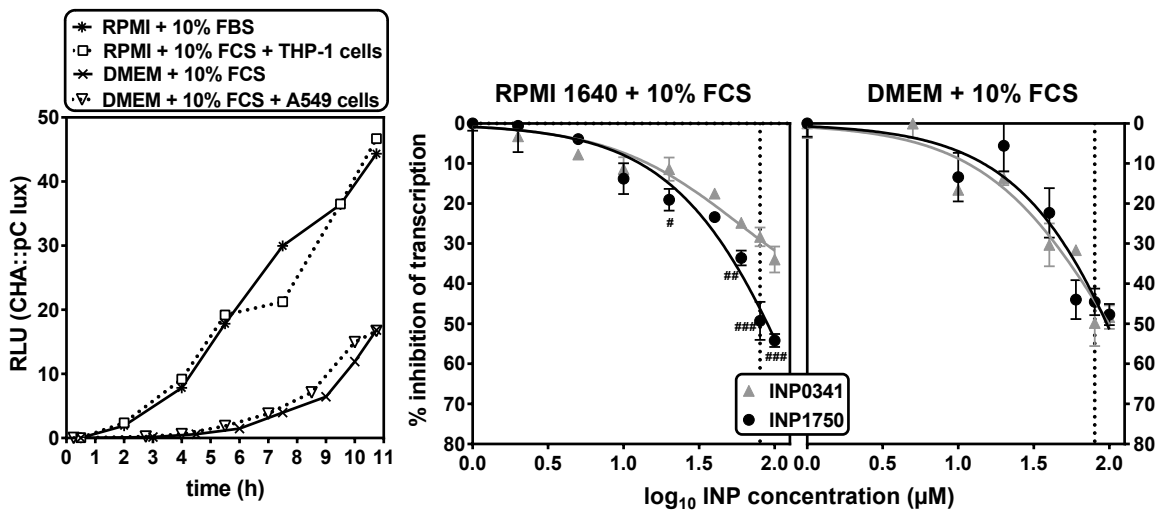
Bacteria were pretreated with 80  $\mu$ M INP0341 or INP1750 or exposed to 0.8 % DMSO during 3 h and then opsonized with human serum during 1 h. Phagocytosis was allowed during 2 h using an initial inoculum of 10 bacteria/cell in medium containing 80  $\mu$ M INP0341 or INP1750 or 0.8 % DMSO, after which non-phagocytosed bacteria were eliminated by incubation with 200  $\mu$ g/mL tobramycin during 1h. Values are mean  $\pm$  SEM of 2 experiments performed in triplicates. Statistical analyses (two-way ANOVA, Bonferroni post-test; comparison between conditions [DMSO, open bars] and cells incubated with INP0341 or INP1750: \*  $p < 0.05$ )



**Figure S5: Influence of INP0341 and INP1750 on medium-dependent *iT3SS* transcription.**

*iT3SS* transcriptional activation of the *exsCEBA* operon was followed by measuring the bioluminescence signal of the reporter strain *CHA pC::lux*. Left: Evolution of bioluminescence over time for bacterial cultured in presence only of eukaryotic cell culture medium (RPMI-1640 + 10 % FCS or DMEM + 10 % FCS; plain lines) or in the presence of THP-1 monocytes (10 bacteria/cell) or of A549 epithelial cells (10 bacteria/cell) in their respective medium. Right: Inhibition of the transcriptional activation of *exsCEBA* operon by increasing concentrations of INP0341 or INP1750 as measured after 5 h of incubation of in the presence of RPMI-1640 + 10 % FCS or after 7 h of incubation in the presence of DMEM + 10 % FCS. Values are expressed in percentage of inhibition of the bioluminescence signal recorded in the absence of inhibitors. All values are mean  $\pm$  SEM of 2 or 3 experiments.

Statistical analyses (two-way ANOVA, Bonferroni post-test; comparison between cells incubated with INP0341 and cells incubated with INP1750): ### $p < 0.001$ , ## $p < 0.01$ .

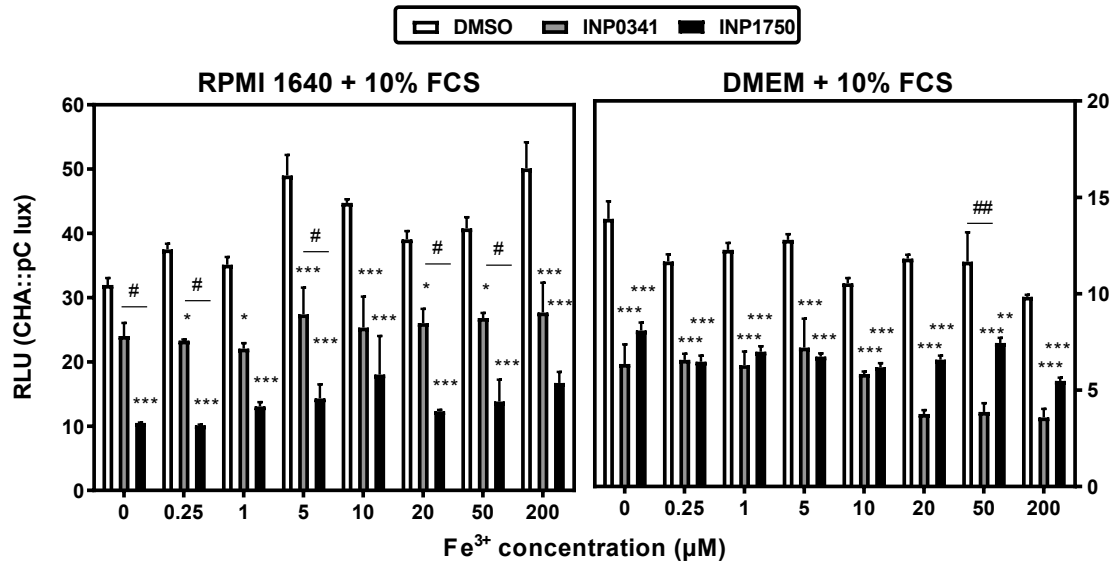




**Figure S6: Influence of culture conditions on *iT3SS* transcriptional activation and its inhibition by INP0341 and INP1750.**

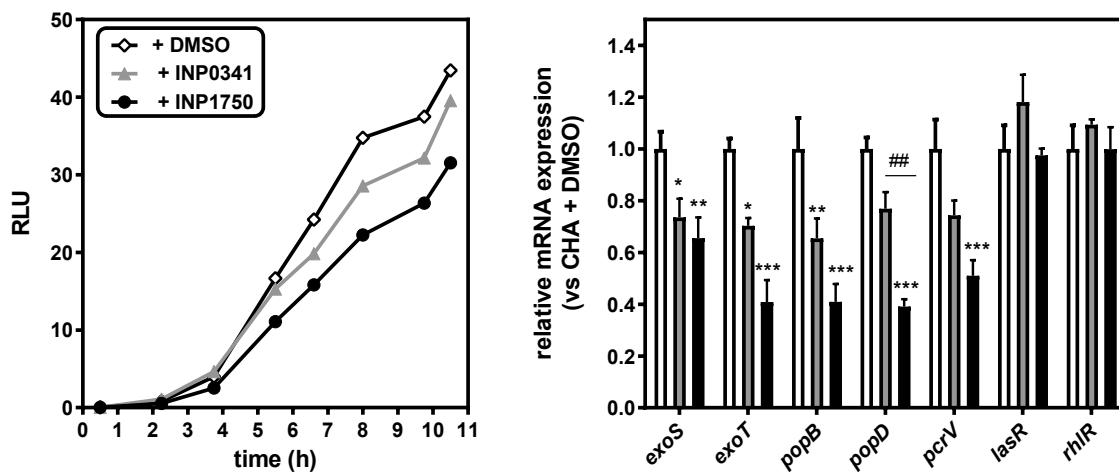
*iT3SS* transcriptional activation of the *exsCEBA* operon was followed by measuring the bioluminescence signal of the reporter strain *CHA pC::lux* after 9 h of incubation in RPMI-1640 or DMEM supplemented by 10 % FCS and increasing concentrations of  $Fe^{3+}$  and in the presence of 0.8 % DMSO (vehicle) or of 80  $\mu M$  INP0341 or INP1750.

Statistical analyses (two-way ANOVA with Bonferroni post-test comparing each of the inhibitors with cells incubated with DMSO; \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$  and comparing the effect of INP0341 and of INP1750 on each concentration: ###  $p < 0.001$ , ##  $p < 0.01$ , #  $p < 0.05$ ).



**Figure S7: Influence of INP0341 and INP1750 on *iT3SS* transcription in low calcium broth.**

Left: Evolution of bioluminescence of the reporter strain CHA *pC::lux* over time in control conditions (0.8 % DMSO) or in the presence of 80  $\mu$ M INP0341 or INP1750 (expressed in arbitrary units [RLU]) for bacteria cultured in low calcium medium (LB broth + EGTA 5 mM + MgCl<sub>2</sub> 20 mM). Right: Influence of INP1855 on mRNA levels of the *iT3SS* toxins (*exoS*, *exoU* and *exoT*), *iT3SS* translocation apparatus (*popB*, *popD*, *pcrV*) or QS transcriptional activator (*lasR* and *rhlR*) genes as determined by real-time PCR. CHA was grown from OD<sub>620 nm</sub> 0.1 to 0.8 with constant shaking in the presence of 0.8 % DMSO (vehicle) or of 80  $\mu$ M of INP0341 or INP1750 in 0.8 % DMSO in low calcium medium at 37°C to stimulate *iT3SS* expression and secretory activity. Results are expressed as relative mRNA expression levels when compared to controls (CHA grown in the absence of inhibitors).



**Figure S8: Influence of INP0341 and INP1750 on pyocyanin production.**

Pyocyanin concentration in the supernatant of bacteria grown overnight with constant shaking in the presence of 0.8 % DMSO (vehicle) or of 80  $\mu$ M of INP0341 or INP1750 in 0.8 % DMSO in LB medium at 37°C. Values are mean  $\pm$  SEM of 4 experiments performed in duplicates.

