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

Authors:

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SUPPLEMENTAL MATERIAL

Table S1 : MICs values and susceptibility patterns[a] of the clinical strains of *Pseudomonas aeruginosa*

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

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

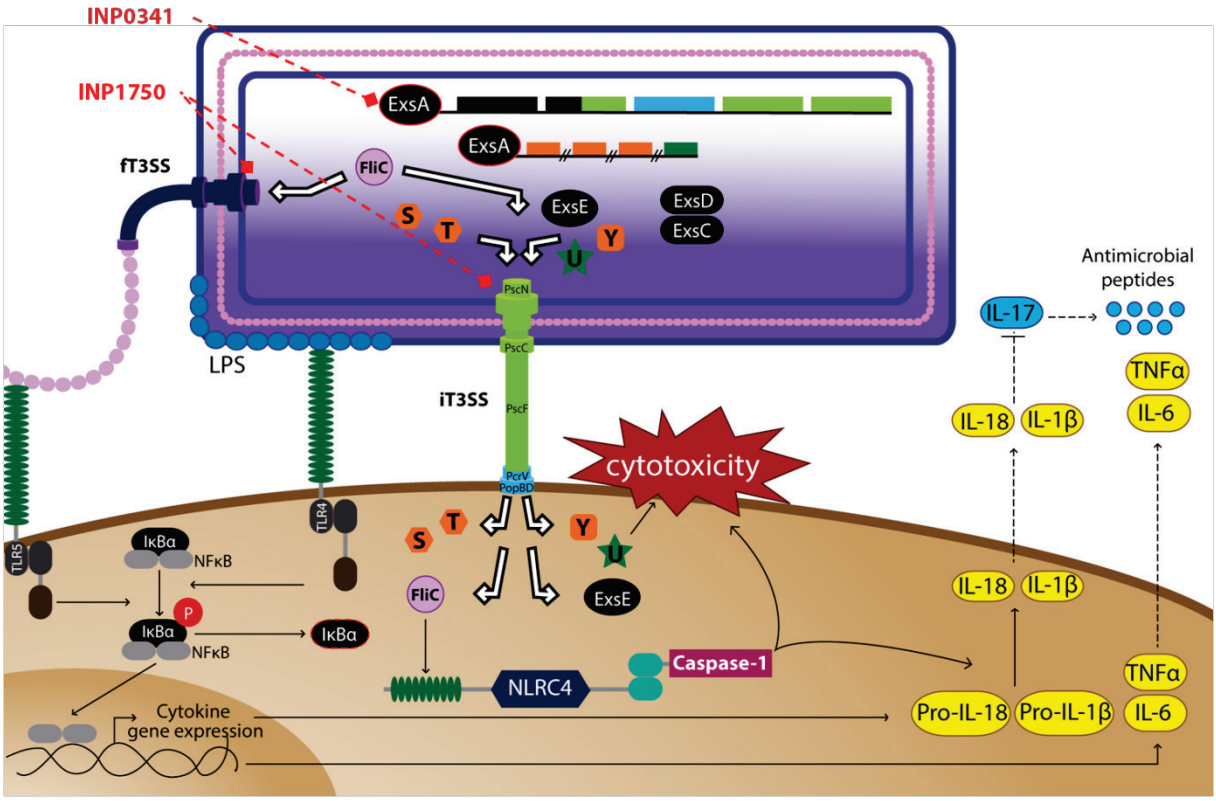
[b] GEN: gentamicin; AMK: amikacin; TOB: tobramycin; MEM: meropenem; ATM: aztreonam; TIC: ticarcillin; PIP: piperacillin: TZP: piperacillin-tazobactam; CAZ: ceftazidime : FEP : cefepime ; CIP : ciprofloxacin ; CST : colistin

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-1β to IL-1β 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 NFκB pathway via TLR4 or TLR5 signaling and phosphorylation of IκBα.

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-1β release. INP1750 also impairs flagellar motility.



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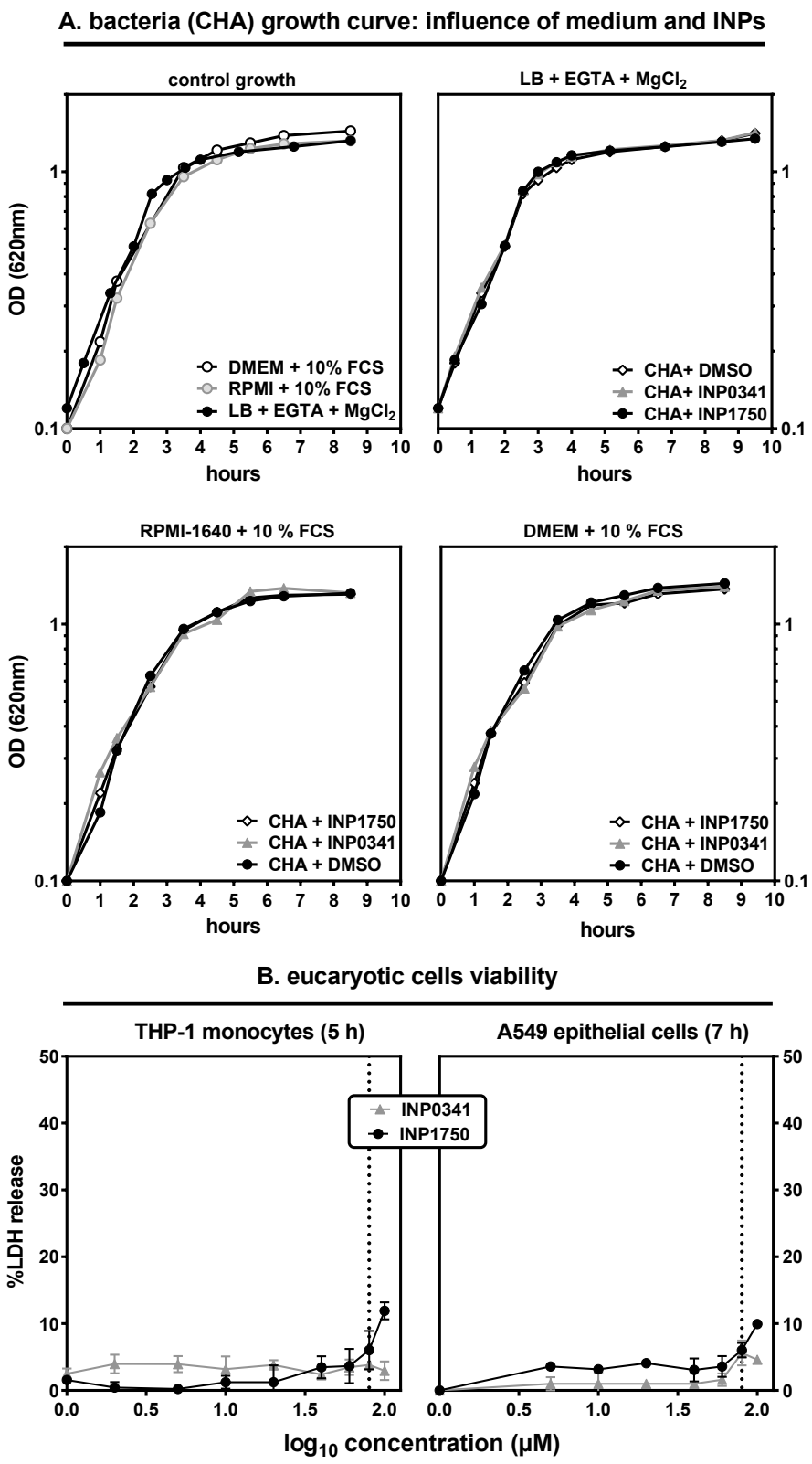

Figure S2: Influence of INP0341 and INP1750 on bacterial growth and on eukaryotic cell viability.

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. 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-α 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 µg/mL), after which cells were centrifuged, washed and incubated during 5 h with different bacterial strains (10 bacteria/cell) or of nigericin (0.02 µM) in the presence of 0.8 % DMSO (vehicle) or 80 µM INP0341 or INP1750 in 0.8 % DMSO. TNF-α was then measured in the supernatant. All values are the mean ± SEM (n=2).

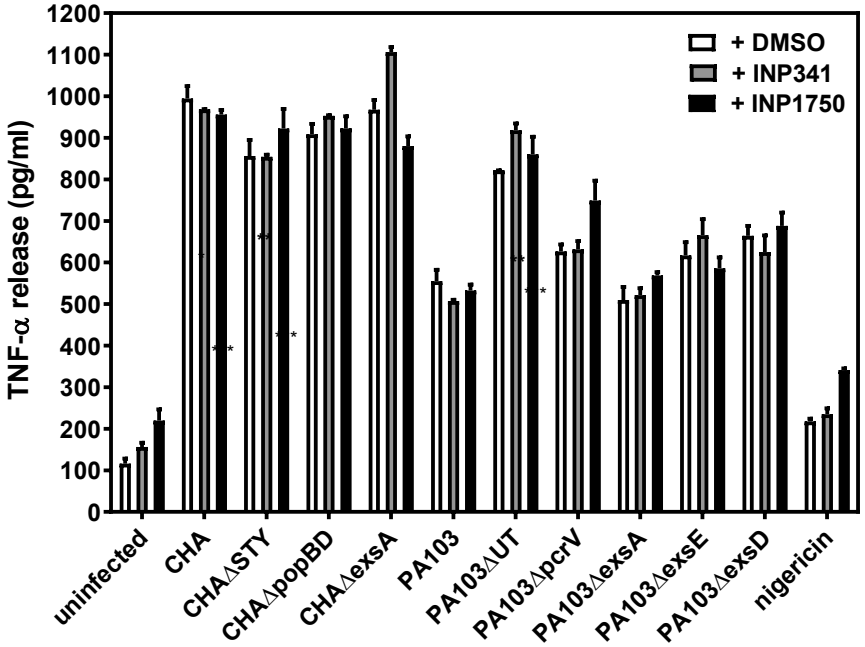

Figure S4: Influence of INP0341 and INP1750 on CHA phagocytosis by THP-1 cells.

Bacteria were pretreated with 80 µM 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 µM INP0341 or INP1750 or 0.8 % DMSO, after which non-phagocytosed bacteria were eliminated by incubation with 200 µg/mL tobramycin during 1h. Values are mean ± 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: * $p<0.05$

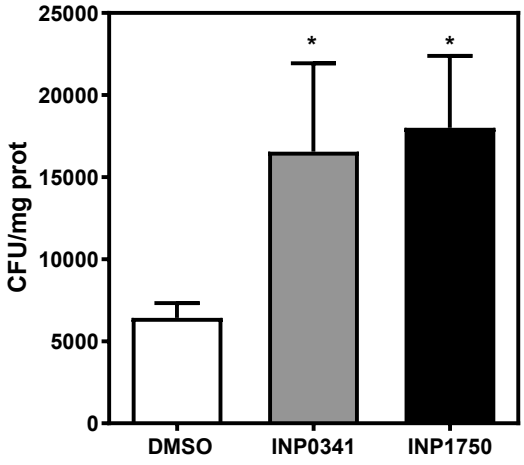

Figure S5: Influence of INP0341 and INP1750 on medium-dependent *iT3SS* transcription.

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 ± 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): $^{###}p<0.001$, $^{##}p<0.01$.

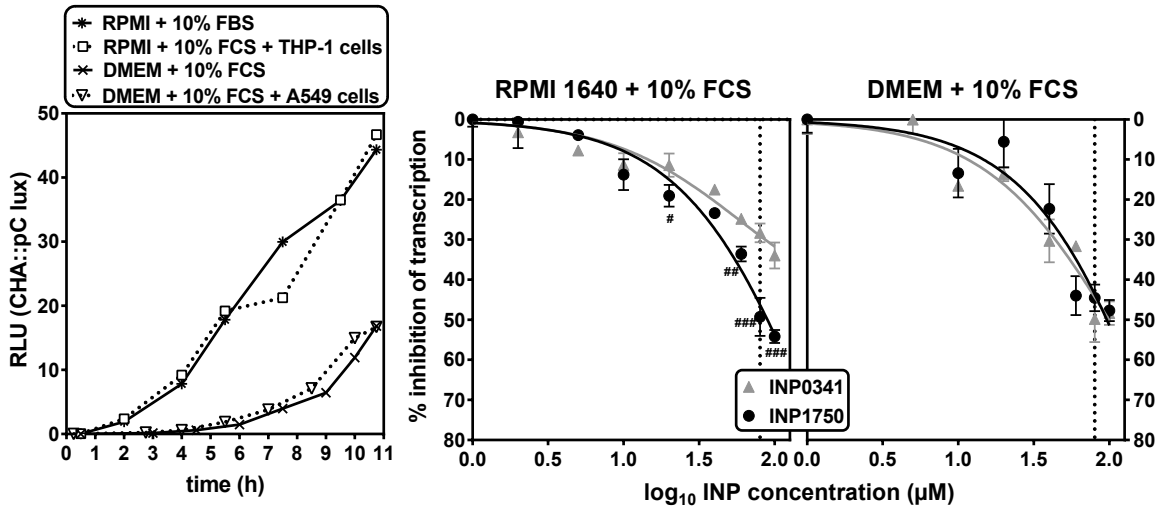

Figure S6: Influence of culture conditions on *iT3SS* transcriptional activation and its inhibition by INP0341 and INP1750.

iT3SS transcriptional activation of the exs*CEBA* 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 µM 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).

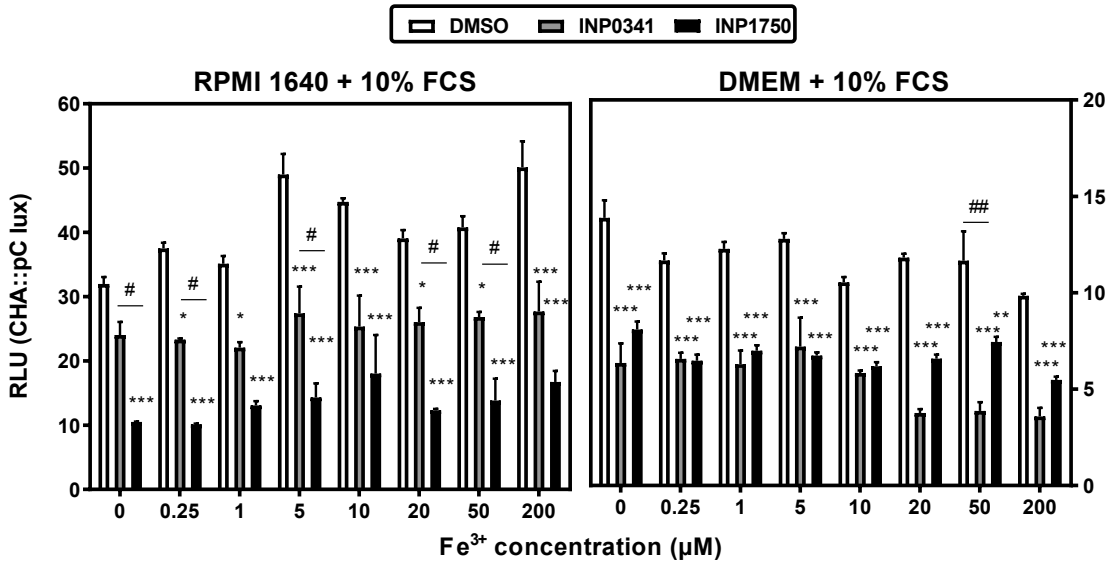

Figure S7: Influence of INP0341 and INP1750 on *iT3SS* transcription in low calcium broth.

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 µM INP0341 or INP1750 (expressed in arbitrary units [RLU]) for bacteria cultured in low calcium medium (LB broth + EGTA 5 mM + $MgCl_2$ 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 $OD_{620\ nm}$ 0.1 to 0.8 with constant shaking in the presence of 0.8 % DMSO (vehicle) or of 80 µM 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).

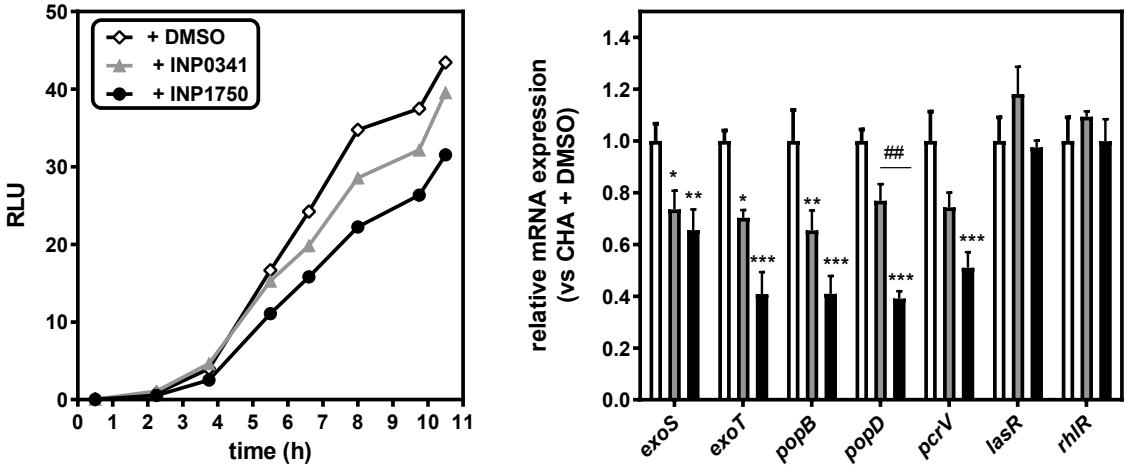

Figure S8: Influence of INP0341 and INP1750 on pyocyanin production.

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