Supplementary Material

Modulation of *Haemophilus influenzae* interaction with hydrophobic molecules by the VacJ/MlaA lipoprotein impacts strongly on its interplay with the airways

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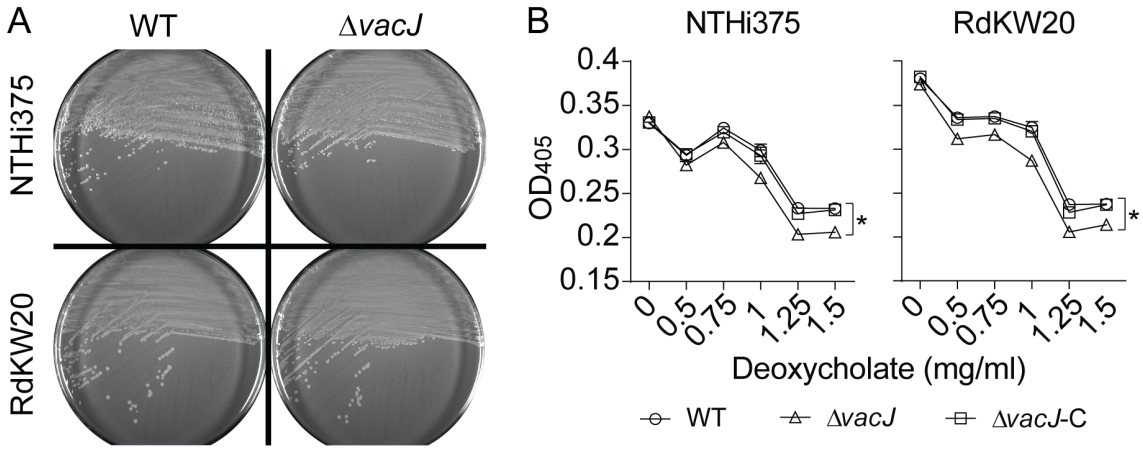

C CLUSTAL O(1.2.4) multiple sequence alignment

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NTHi375           ATGAATACAAAAATGCTATGCAATCAATCAATCAATCAATCAATCAATCAATCAATCAAT
NTHi375ΔvacJ      ATGAATACAAAAATGCTATGCAATCAATCAATCAATCAATCAATCAATCAATCAATCAAT
RdKW20            ATGAATACAAAAATGCTATGCAATCAATCAATCAAT----------CAAT
RdKW20ΔvacJ       ATGAATACAAAAATGCTATGCAATCAATCAATCAAT----------CAAT
                  ************************************          ****

NTHi375           CAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATTGTAGGATTTG
NTHi375ΔvacJ      CAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATTGTAGGATTTG
RdKW20            CAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATTGTAGGATTTG
RdKW20ΔvacJ       CAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATTGTAGGATTTG
                  ************************************************************

NTHi375           TTAAAACTTGCT
NTHi375ΔvacJ      TTAAAACTTGCT
RdKW20            TTAAAACTTGCT
RdKW20ΔvacJ       TTAAAACTTGCT
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	NTHi375		RdKW20	
H_2O_2 (μmol)	WT	Δ*vacJ*	WT	Δ*vacJ*
3.15	1.68 ± 0.1	1.70 ± 0.1	0.67 ± 0.1	0.63 ± 0.1
6.25	2.07 ± 0.2	2.07 ± 0.2	0.80	0.77 ± 0.1
12.5	2.48 ± 0.2	2.47 ± 0.2	1.00	1.00
25	2.88 ± 0.3	2.97 ± 0.4	1.43 ± 0.1	1.30

Figure S1. (A) Morphology of bacterial colonies rendered by NTHi375 and RdKW20 WT and *vacJ* mutant strains, grown on chocolate agar for 16 h at 37ºC with 5% CO_2. Colony size and morphology was comparable between WT and mutant strains. **(B)** Sodium deoxycholate bactericidal effect on NTHi depends on VacJ. NTHi WT and Δ*vacJ* strains grown on chocolate agar were used to generate OD_{600}-normalized bacterial suspensions for further incubation with sodium deoxycholate. After 20 min, deoxycholate dose dependent bactericidal effect was higher on the Δ*vacJ* mutant than on the WT strains, as measured by assessing turbidity (for NTHi375, $p<0.005$; for RdKW20, $p<0.0005$) (WT strains, circle;

Δ*vacJ* mutants, triangle; Δ*vacJ* complemented strains, square). Phenotypic restoration in Δ*vacJ* complemented strains (Δ*vacJ*-C) could be observed. Experiments were performed in quadruplicate, in three independent occasions (n=12). Results are shown as means ± SE; statistical comparisons were performed with two-way ANOVA (Tukey's multiple comparisons test). **(C)** Multiple sequence alignment of the 5´end of the *lic1A* gene containing (5'-CAAT)$_n$ tetranucleotide repeats. Primers Lic1prF1-HindIII/858 (5'-GCGGATTATTACAATCTATTTAACCTC) and Lic1ART-Rv/1099 (5'-AAGTAGAACATTTTGATTGGTCATTCC) were used for DNA amplification using NTHi375 and RdKW20 WT and *vacJ* mutant strains as template, and Sanger sequencing. The *vacJ* gene inactivation did not vary the number of repeats, i.e. 21 in NTHi375 and 16 in RdKW20 strains. **(D)** Effect of H_2O_2 on NTHi growth inhibition, measured by disc diffusion assay. NTHi cells were scraped from freshly grown chocolate agar plates into PBS, and adjusted to OD_{600}=1. Normalized suspensions containing ~7x10^8 c.f.u./ml were plated on sBHI agar. H_2O_2 9.8 M (Sigma-Aldrich) was used as stock solution to be further diluted in sterile distilled water. Sterile paper discs soaked on 10 μl H_2O_2 containing 3.15, 6.25, 12.5 or 25 μmol were located on the sBHI agar and plates were incubated. Results are shown as diameter of bacterial growth inhibition (cm). Experiments were performed three times (n=3). No significant differences were observed between WT and mutant strains by means comparison with two-tail *t*-test.

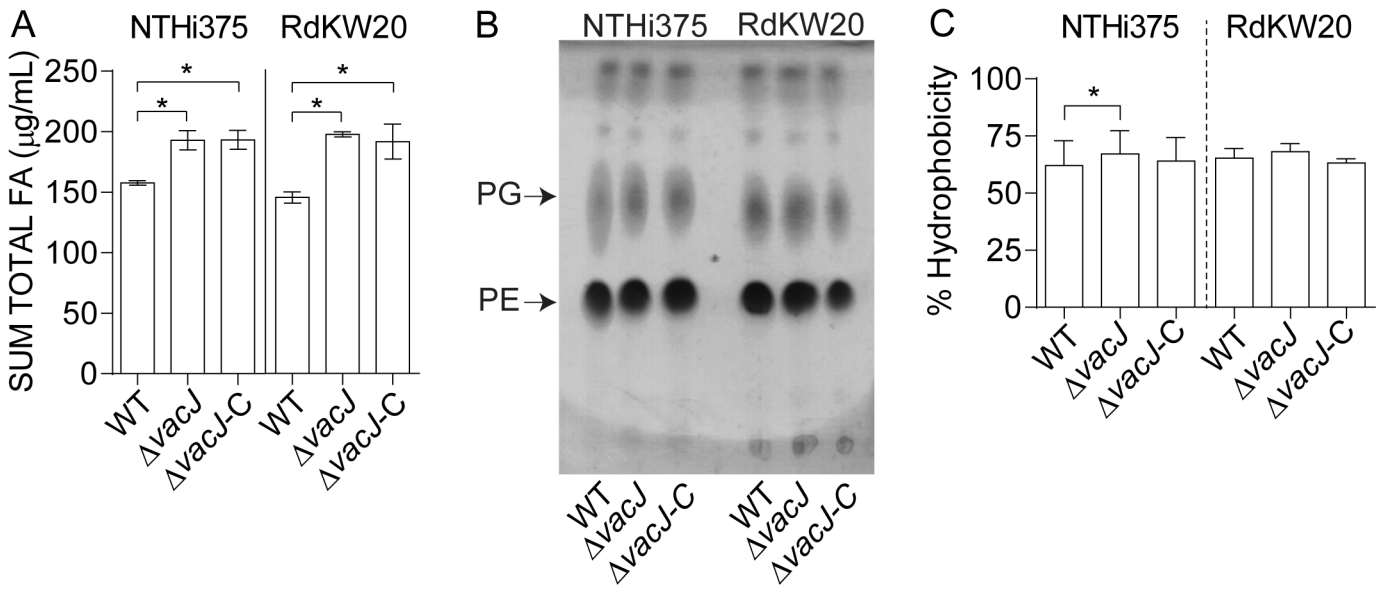

Figure S2. Total fatty acids and phospholipids in NTHi WT and *vacJ* mutant strains. **(A)** Total fatty acid composition, as methyl esters (shown as means ± SE). Bacteria grown on chocolate agar were used to extract fatty acids, by following saponification, methylation, extraction and washing steps. *vacJ* mutants showed increased fatty acid amounts (for NTHi375 and RdKW20, $p<0.005$). Phenotypic alterations were not restored in the *vacJ* complemented strains (Δ*vacJ*-C) (for NTHi375 and RdKW20, *$p<0.005$). Measures were performed in duplicate, in three independent occasions (n=6). Two-way ANOVA (Sidak's multiple comparisons test) was used for statistical analysis. **(B)** TLC bands corresponding to PE and PG phospholipids, in NTHi375 and RdKW20 WT, Δ*vacJ* and Δ*vacJ* complemented strains. **(C)** Bacterial surface hydrophobicity. Bacteria grown on chocolate agar were processed to measure their hydrophobicity (shown as means ± SD). Bacterial surface hydrophobicity showed a trend to be higher for Δ*vacJ* (NTHi375Δ*vacJ*, 67.2±10.2%; RdKW20Δ*vacJ*, 68.2±3.5%) than for their isogenic WT strains (NTHi375, 62.1±10.9%; RdKW20, 65.4±4.1%). Hydrophobicity rendered values of 64±10.4 and 63.3±1.7% for NTHi375Δ*vacJ*-complemented and RdKW20Δ*vacJ*-complemented strains. Surface hydrophobicity was higher for Δ*vacJ* than for NTHi375 WT strain (*$p<0.0005$).

Measures were performed in duplicate, in three independent occasions (n=6). Two-way ANOVA (Sidak's multiple comparisons test) was used for statistical analysis.

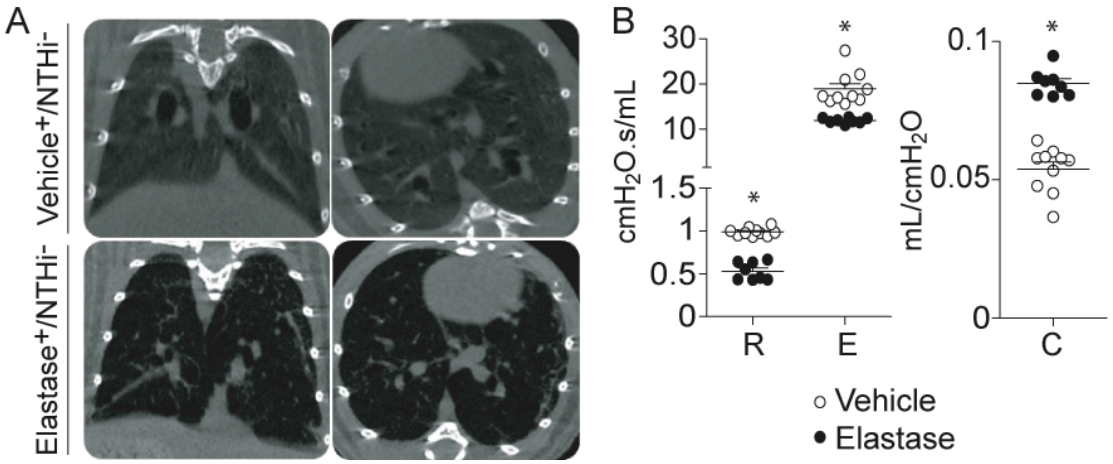

Figure S3. Elastase instillation induces a lesion compatible with lung emphysema in CD1 mice (n≥8 per group). Emphysema was induced by intratracheal instillation of 6 U/mouse of porcine pancreatic elastase. In parallel, control animals were instillated with vehicle solution. Lung emphysema was evaluated 17 days after elastase/vehicle administration using X-ray Micro-CT **(A)** and pulmonary function tests (PFT) **(B)**. **(A)** Representative images for longitudinal (left panels) and transversal (right panels) micro-CT slices are shown. Elastase-treated animals presented lower tissue density and abnormally expanded lungs compared to vehicle animals. **(B)** Elastase-treated animals (black circles) showed higher compliance (C), lower resistance (R) and lower elastance (E) than vehicle-treated animals (white circle). Means ± SD are shown, compared by *t*-test, *p<0.0005.

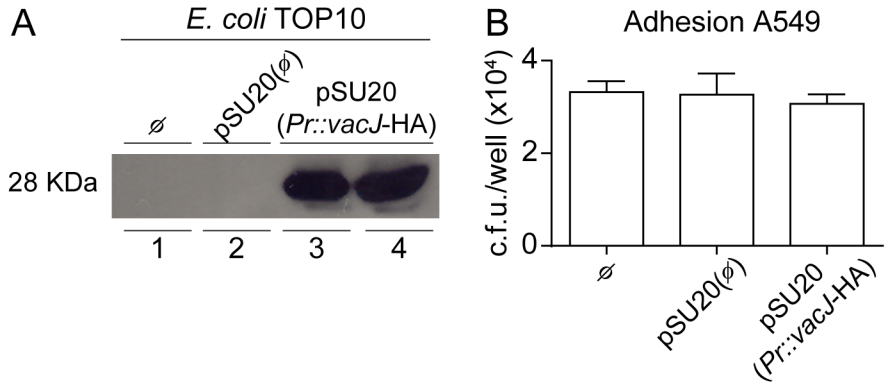

Figure S4. Heterologous expression of the $vacJ_{NTHi}$ gene in *E. coli* TOP10 does not modulate bacterial epithelial adhesion. **(A)** The $vacJ_{NTHi375}$ gene with a HA tail in its 3´end, together with its predicted promoter region, were PCR amplified using NTHi375 genomic DNA as template and primers vacJ-F1/931 (5'-ATGACTGAAGAAACAACPAGTATCAAAA) and *Pr-vacJ*-HA-R/1440 (5'-GATGACATACTAAAAGAAATTGATTACCCATACGACGTCCCAGACTACGCTTAA). The 1,487 bp PCR product was cloned into Hinc*II* digested pSU20, generating pSU20-*Pr::vacJ*-HA. $VacJ_{NTHi}$ protein was monitored in *E. coli* TOP10 by western blot. To this end, whole cell extracts were prepared from bacterial suspensions recovered from LB+Cm 30 μg/ml cultures, adjusted to OD_{600}=1 in PBS, lysed by ultrasounds sonication, two-fold diluted with 2X loading buffer (Tris-HCl 62.5mM pH 6.8, SDS 2% w/v, glycerol 10%, DTT 50 mM; Bromophenol Blue 0.01% w/v), and heated to 95ºC for 5 min. Proteins were separated on 12% SDS-PAGE gels (BioRad, MINI PROTEAN Tetra System) and transferred to a nitrocellulose membrane. VacJ protein (~28 KDa) was monitored with primary rabbit anti-HA antibody (Sigma-Aldrich) diluted 1:4000, and secondary goat anti-rabbit IgG (whole molecule, Sigma-Aldrich) antibody conjugated to horseradish peroxidase, diluted 1:1000. Lane 1, untransformed bacteria; lane 2, bacteria transformed with empty pSU20 vector; lanes 3 and 4, bacteria transformed with pSU20-*Pr::vacJ*-HA, two clones were tested. **(B)** A549

cells were infected with *E. coli* TOP10 (MOI~50:1) to quantify adhesion (means ± SD are shown). After infection (30 min at 37ºC and 5% CO_2), wells were washed three times with PBS and incubated with saponin 0.025% during 10 min for cell lysis. Lysates were 10-fold serially diluted in PBS and plated on LB agar. Adhesion assays were performed in triplicate, in three independent occasions (n=9). No significant differences were observed (two-tail *t*-test).

Table S1. Minimal inhibitory concentration (MIC) of 12 hydrophilic antibiotics against NTHi WT and *vacJ* mutant strains by the microdilution method.

	MIC (mg/l)											
NTHi strain	**Ampicillin**	**Amoxicillin/ Clavulanic**	**Cefuroxime**	**Cefepime**	**Cefotaxime**	**Ceftriaxone**	**Imipenem**	**Meropenem**	**Chloranfenicol**	**Tetracycline**	**Ciprofloxacin**	**Cotrimoxazole**
NTHi375 WT	0,25	<0,5/0,25	2	<0,25	<0,06	<0,12	1	<0,25	<1	<1	<0,03	<0,5/9,5
NTHi375Δ*vacJ*	0,25	<0,5/0,25	2	<0,25	<0,06	<0,12	0,5	<0,25	<1	<1	<0,03	<0,5/9,5
RdKW20 WT	0,25	<0,5/0,25	2	<0,25	<0,06	<0,12	0,5	<0,25	<1	<1	<0,03	>2/38
RdKW20Δ*vacJ*	0,25	<0,5/0,25	1	<0,25	<0,06	<0,12	0,5	<0,25	<1	<1	<0,03	>2/38

Table S2. *H. influenzae* minimal medium free of fatty acids (MM-FFA): composition and preparation.

S21 (500 ml):		**S22 (100 ml):**		**S23 (100 ml):**	
L-Aspartic Acid	2 g	L-Cystine	0,04 g	$CaCl_2$	1,1099 g
L-Glutamic Acid	0,1 g	L-Tyrosine	0,1 g	dH_20	100 ml
Fumaric Acid	0,5 g	Dissolve in 10 ml of HCl 1N		Autoclave.	
NaCl	2,35 g	at 37ºC.		Store at 4ºC.	
K_2HPO_4	0,435 g	Add dH_2O M-Q to 100 ml.			
KH_2PO_4.	0,335 g	Add to this mixture:			
Tween80	0,1 ml	L-Citruline	0,06 g	**S24 (100 ml):**	
Ajust pH to 7.4 with NaOH 4N.		L-Phenylalanine	0,2 g	$MgSO_4$	2,4648 g
Add dH_2O to 500 ml.		L-Serine	0,3 g	dH_20	100 ml
Autoclave and store at RT.		L-Alanine	0,2 g	Autoclave.	
		Sterilize by filtration.		Store at 4ºC.	
		Store at 4ºC.			

MM-FFA preparation:

1) Mix S21:S22:S23:S24 (volume rate, 100:1:1:1)
2) Supplement the mix with hemin (10 μg/ml) and β-NAD (10 μg/ml)
3) If necessary, add glucose 20 mM (stock solution: glucose 1M prepared in distilled water and sterilized by filtration)

Table S3. Distribution and level of conservation of genes involved in fatty acid uptake, degradation and recycling in *H. influenzae* (protein BLAST).

	***E. coli* gene**	**Gene ID**	**Protein ID**	**Hi gene**	**Gene ID RdKW20**	**Protein ID RdKW20**	**% identity *E. coli***	**Gene ID NTHi375**	**Protein ID NTHi375**	**% identity *E. coli***
Fatty acid degradation	*fadL*	b2344	NP_416846.2	*fadL*	HI0401	NP_438563.1	40%	NF38_03050	AIT67229.1	39%
	fadD	b1805	NP_416319.1	*fadD*	HI0390.1	NP_438551.1	65%	NF38_03105	AIT67240.1	65%
	fadE	b0221	NP_414756.2	---	---	---	---	---	---	---
	fadB	b3846	NP_418288.1	---	---	---	---	---	---	---
	fadA	b3845	YP_026272.1	---	---	---	---	---	---	---
Membrane lipid asymmetry maintenance	*mlaA*	b2346	NP_416848.1	*vacJ*	HI0718	NP_438876.1	42%	NF38_01540	AIT66950.1	42%
	mlaB	b3191	NP_417658.4	*mlaB*	HI1083	NP_439240	32%	NF38_08695	AIT68260	32%
	mlaC	b3192	NP_417659.1	*mlaC*	HI1084	NP_439241	42%	NF38_08690	AIT68259	43%
	mlaD	b3193	NP_417660.1	*mlaD*	HI1085	NP_439242	60%	NF38_08685	AIT68258	58%
	mlaE	b3194	NP_417661.1	*mlaE*	HI1086	NP_439243	73%	NF38_08680	AIT68257	72%
	mlaF	b3195	NP_417662.1	*mlaF*	HI1087	NP_439244	67%	NF38_08675	AIT68256	67%
	pqiA	b0950	NP_415470.1	*pqiA*	HI1671	NP_439813.1	37%	NF38_05885	AIT67735.1	37%
	pqiB	b0951	NP_415471.1	*pqiB*	HI1672	NP_439814.1	43%	NF38_05880	AIT67734.1	43%
Outer membrane phospholipase	*pldA*	b3821	NP_418265.1	---	---	---	---	---	---	---
Acyl-transferases	*lpxA*	b0181	NP_414723.1	*lpxA*	HI1061	NP_439219.1	68%	NF38_08800	AIT68281.1	68%
	lpxD	b0179	NP_414721.1	*lpxD*	HI0915	NP_439075.1	65%	NF38_00400	AIT66742.1	65%
	lpxL	b1054	NP_415572.1	*htrB*	HI1527	NP_439676.2	55%	NF38_07450	AIT68026.1	57%
	msbB	b1855	NP_416369.1	*msbB*	HI0199	NP_438368.1	45%	NF38_04430	AIT67476.1	45%
	plsB	b4041	NP_418465.4	*plsB*	HI0748	NP_438907.1	57%	NF38_ 01210	AIT66899.1	57%
	plsC	b3018	NP_417490.1	*plsC*	HI0734	NP_438893.1	62%	NF38_ 01275	AIT66912.1	62%
	pagP	b0622	NP_415155.1	---	---	---	---	---	---	---