

Supplementary Information

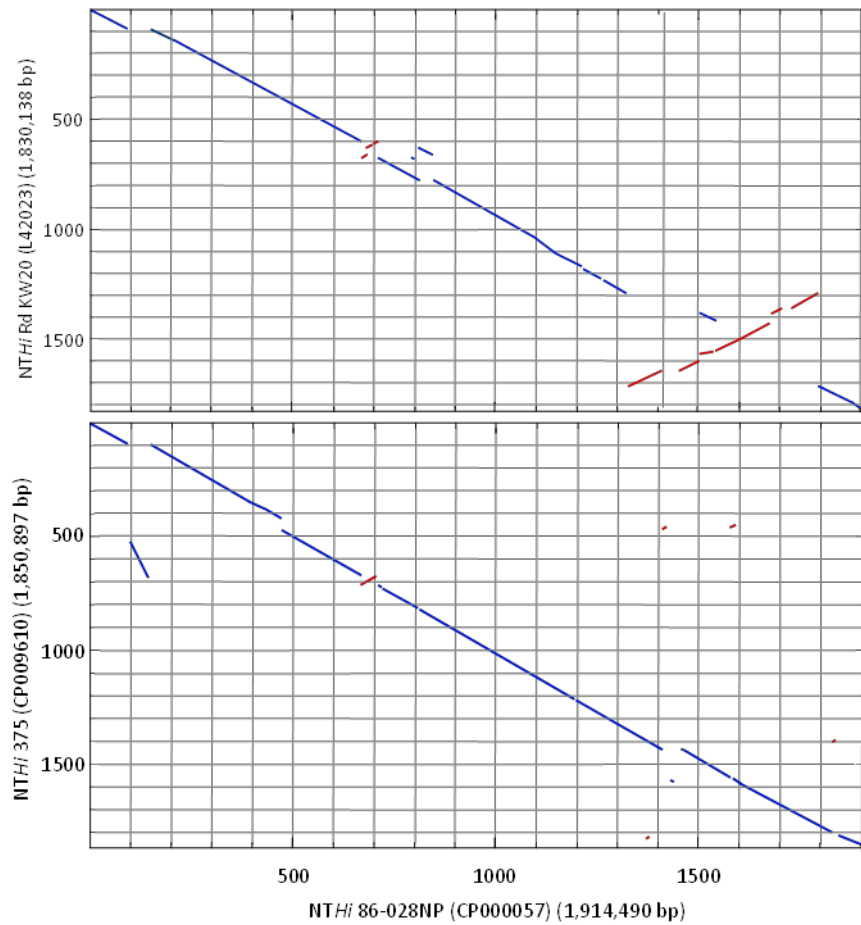
Evidence of the presence of nucleic acids and β -glucan in the matrix of non-typeable *Haemophilus influenzae in vitro* biofilms

Mirian Domenech^{1,3}, Elena Pedrero-Vega¹, Alicia Prieto² and Ernesto García^{1,3}

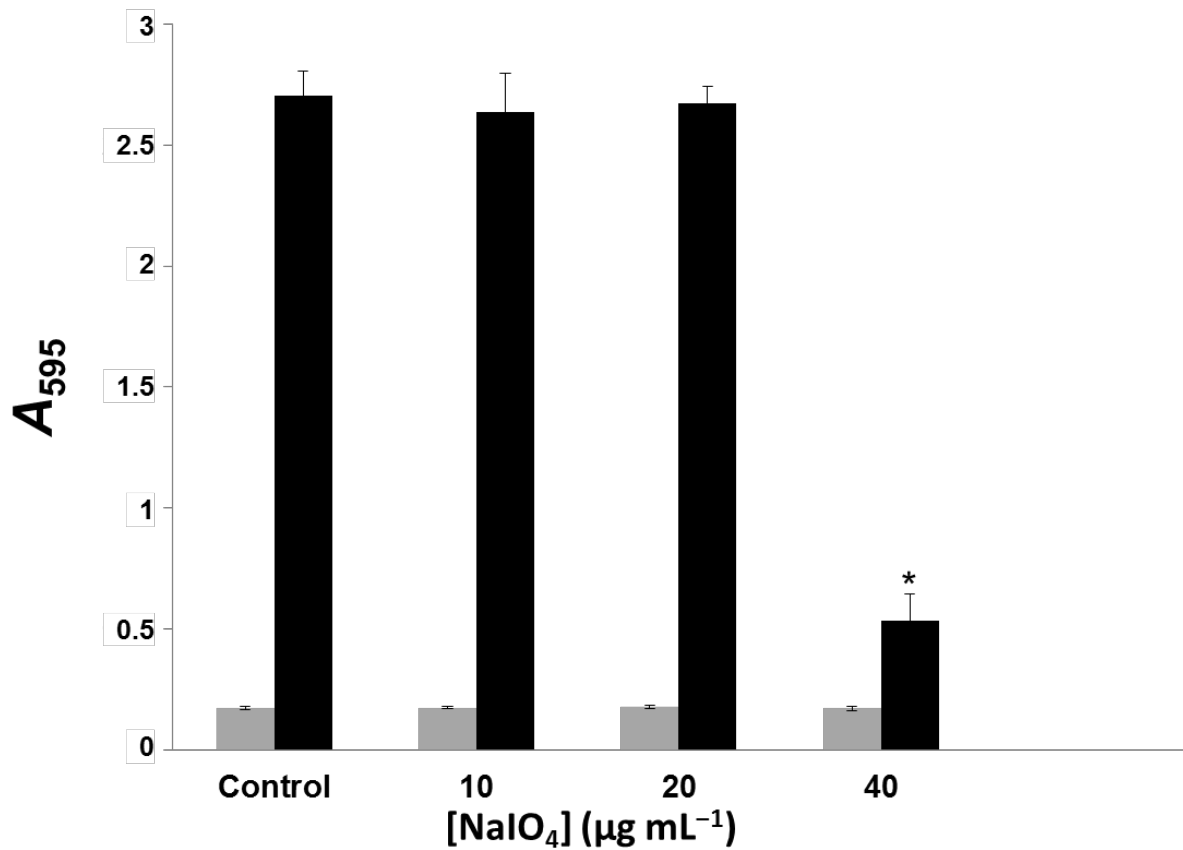
¹Departamento de Microbiología Molecular y Biología de las Infecciones, and

²Departamento de Biología Medioambiental, Centro de Investigaciones Biológicas (CSIC), Ramiro de Maeztu 9, 28040 Madrid, Spain. ³CIBER de Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III, Monforte de Lemos 3–5, 28029 Madrid, Spain.

Correspondence to: Mirian Domenech [mirid@cib.csic.es; Tel. (+34) 918 373 112; Fax (+34) 915 360 432].



Supplementary Figure S1. Dot slot matrices calculated for the genome sequences of three non-encapsulated *H. influenzae* strains. The genome alignment of strains Rd KW20 and 86-028NP served as a control¹. As nucleotide position 1 of the genomic sequence of strains Rd KW20 and 86-028NP corresponds to nucleotide 1,099,336 of the reverse complement of that of strain 375 (Hi375) (Acc. No. CP009610)², the latter sequence was rearranged accordingly to match the genome of the other two strains. The dot matrices were calculated using wgVISTA software³. The diagonal lines in the image show the homologous regions between the compared genomes. Blue and red lines indicate that the regions are on the same strand, or on opposite strands, respectively.



Supplementary Figure S2. Disaggregation of a *NTHi* 54997 biofilm by NaIO_4 . Cells of *NTHi* 54997 were distributed in the wells of a microtiter plate, which was then incubated for 7 h at 37 °C in a 5% CO_2 atmosphere. Grey bars indicate growth (adherent plus non-adherent bacteria). Biofilms were washed with sterile water and incubated with NaIO_4 for 2 h at 37 °C under 5% CO_2 . Biofilm formation was quantified by staining with CV (black bars). * $P < 0.001$ compared to the control.

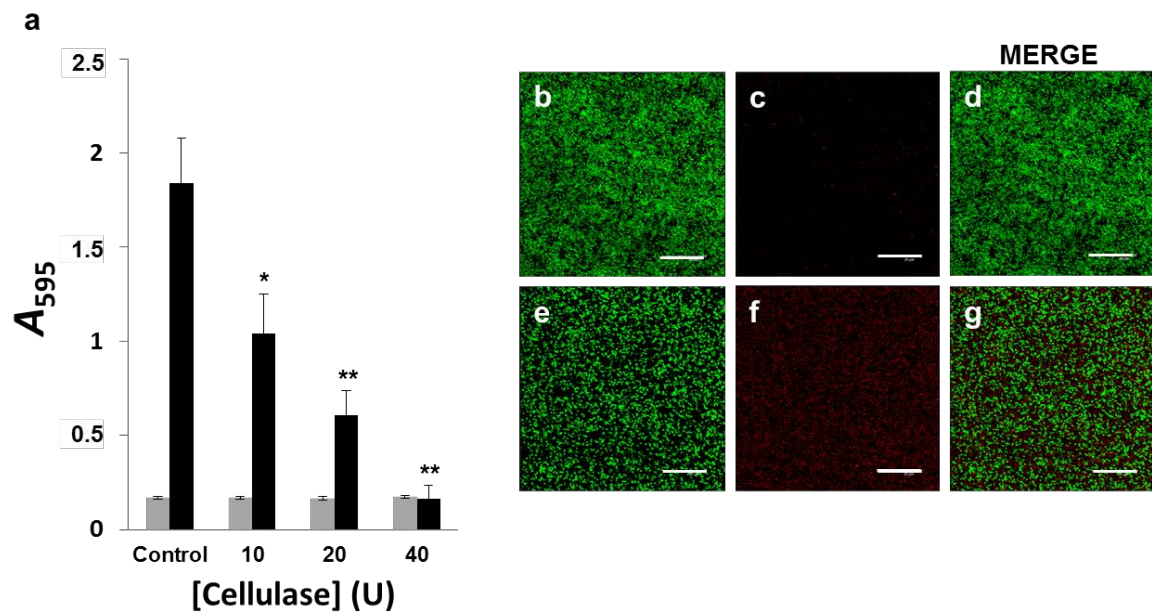
a

AAA20527	SGHDLFIKDNAIVDAKEWLLDPDNVSINAETAGRSNISEDEYTGSGNSASTPKRNEK-	479
AAx88733	SGHYLSIGNDAAVEAKEWLLDPDNVTISNGNDQSQLKDDRG-----DSPNKILADNK	472
AIT67726	SGHYLSIDSNAIYKTEWLLDPDNVTIEAPSSSRGNAGIDSEFPGGSGTKESPKTNGEQP	480
AAA20527	TTLTNTLESILKKGTFV-NITANQRIYVNSSINLSNG-SLTLSWSEGRSGGGVEINNDIT	537
AAx88733	HIVNNKTLSTALAKGIGV-NISAKKKVNVITADINVHNG-TLTLSH---EQGGVEINGDIT	527
AIT67726	TVLTNETISNYLKSIGIWMNITAKKNLTVNSSINIGDSSHLLHSEKNGGVIKEDIT	540
AAA20527	TGDDIRGANLTIYSGGWVDVHKNI SLGAQGNINI TAKQDIAFEKGSN-----QVITG	589
AAx88733	SE---QNGNLIIKAGSWVDVHKNIITGM-GFLNI TAGGSVAFEKAGGDKGRAASDAKIVA	583
AIT67726	S---NGGNLIIQSGGWVDVHKNIITLGT-GTLNITAGKSIAFENGTEKARNASSAQITA	595
AAA20527	QGTI-TSGNQKGFRRFNNVSLMTGSGLQFTTKRTNKYAITNKFEGTLNISGKVNI SMVLP	648
AAx88733	QGTI-TAGSGQDFRRFNNVSLMTGSGRLFTI TAKNGKGNFSAKFDGVLNISGNI SINHTAN	642
AIT67726	QGTITNTGQDLRLNNVSLMTGIGLNFVSIQPN---TSHRFDGLIISGRVHVNQITP	652
AAA20527	KNESGYDKFKGRITYWNLITSLNVSESGEFNLII DSRGSDSAGTLIQ-PYNLN-GISFN--	703
AAx88733	NQLS-YFHRQSYTYWNLITQLNVSDSDSFLTSIKDAIKVGSYDIAKDKKNTGGIGFT--	698
AIT67726	KNLS-FWKVSDESYWNVSHLTVKEKSAFSTFKFALNNH-GRETS-RYRKGGGVI FRSP	709
AAA20527	KDITFNVERNARVNFIDIKAPIGINKYSSLNYSFNGNISVSGGGSVDFTLASSSNVQTP	763
AAx88733	RDITFNVKQGARVDISYTLPI SFYKNSRAAVNEDGNI TVKGGGVNLFKFNALSNVKT	759
AIT67726	GHTNFVVKGSVANFSAKNDTNHANQ-LPIQNSNI SVGGGKVLFCITISN---ISGR	765
AAA20527	GVVINSKYFNVSIGSSIRFKTSGSTKTGFSIEKDLTINATGONITLLQVEGT---DGMIG	820
AAx88733	GVNISSRFNVITEGSQLNITGSMPTTLFNVANDLI INATNSFVSIKEIEGT---DTHLD	815
AIT67726	SVGIGMSSLINVSIGSNIITFNSSIRGQEA FNISKDLTINATGSGFFEGLQYSDTFMNGFNH	825
AAA20527	KGIVAKKNITFEAGNITFGSRKAVTEIEGNVI INNNANVT LIGSDFDNHOKPLTIKKDVI	880
AAx88733	TGLKVNIGNVTIKGGNVILGSKNAKTKFDKNVIVEKGANLT LASANFGNKGALTVAG-NI	874
AIT67726	DAIKSANNISILGGNVILGGQSSSTITGNINISQAANVILRAYNGNGRNKQLTLG-NV	883
AAA20527	INSGNITAGGNIVNIAGNLITVE SWANFKAI NTFN FNVGGLFDNKGNSNI SIAKGGARFKD	940
AAx88733	NTQCKLVATGDTIDVSGDFTVGNDAITFNGNTNNNLNITGNFTNNGTSIIDVKKGAACKLN	934
AIT67726	SIEGNLSLIGASANINGNLSVKENAKFKGTQDNLNITGTFINNGSKINISQGVVKLGN	943
AAA20527	IDNSKNLSITINSSSTYRIIISGNITNKNGDLNITNEGSDTEMQIGGDSVQKEGNLTISS	1000
AAx88733	ITNEGSLNITTHANTNOKTIIITGNITNKNGDLNIRDNKNNAEIQIGNISQKEGNLTISS	994
AIT67726	VINDGDLNITTHAKHNQSIIGGDIINKKGLNITDSNKNAEIQIGNISQKEGNLTISS	1003
AAA20527	DKINITKQITIKAGVDGENSDSDATNNANLTIKT KELKLTQDLNISGFNKAETAKDGSD	1060
AAx88733	DKVNITKQITIKAGVNGENSDSGTENNANLTIKT KTLTLNINLISGFNKAETAKDNSD	1054
AIT67726	DKNITINQITIKAGVDGENSDSDATNNANLTIKT KELKLTQDLNISGFNKAETAKDGSD	1063
AAA20527	LIIGNTNSADG-TNAKKVIFNQVKDSKI SADGHKVTILHSHKVTESG-SNNNTEDSSDNAG	1118
AAx88733	LIIGKASSDSGNAGAKVIFDKVKDSKI SAGDHNVITLSEVETSGNNSHAGDSNGNAG	1114
AIT67726	LIIGNTNSADG-TNAKKVIFNQVKDSKI SAGDHNVITLSEVETSGNNTIDNTEGDSGNAG	1121
AAA20527	LTIDAKNVIVNNNITSHKAVSISATSGEITTKTGTTINATIGNVEITATGSGILGDISS	1178
AAx88733	LTISAKDVAVNNNITSHKTIINISATTGNVTIKEGTTINATIGGVEVIATGDIKGGIESK	1174
AIT67726	LTIAAKNVVNNNITSNKIVNIAS-EKLTIKADATINATIGNVEVIATGDIKGEVKST	1180
AAA20527	SGSVITLITATEGALAVSNI SGNTVIVTANSGALITLAGSTIKGTESVITSSQSGDIGGTIS	1238
AAx88733	SGGVITLITATGDTLAVGNI SGNTVSVTANSGTLITKADSTIKGTGSVITLSSQSGDIGGTIS	1234
AIT67726	SGNVNITANGDTLNVSNVSGNAVITAD-----KADSTIKGTGSVITLSSQSGDIGGTIS	1208
AAA20527	GGTVEVKATESLITQSNISKI KAITGEANVT SATGTIGGTISGNTVNVNANAGDLTVNGA	1298
AAx88733	GKTVS-----VTATDSLTVKGA	1253
AIT67726	GKTVS-----VTATDSLTVKGA	1208
AAA20527	EINATEGAATLTSSGKLITEASSHITSAGQVNLSDQGSVAGSINAANVILNTTGTLT	1358
AAx88733	KINATEGATLTASGKLITEASSSITSAGQVNLSDQGSVAGSINAANVILNTTGTLT	1313
AIT67726	-----KGKLITQASSSITSNNGTTLTAKDGS IAGSINAANVILNTTGTLT	1254
AAA20527	TVKGSINATSGTLVINAKDAELNGAALGNHTVVNATNANGSGSVIATTSRVNITGDLI	1418
AAx88733	TVKGSINANSGTLVINAEADAKLDGTASGDRTVVNATNANGSGSVIATTSRVNITGDLI	1373
AIT67726	TVEGSINAAAGSLTIVINAKDAELNGAASGDHTVVNATNANGSGSVIATTSRVNITGDLI	1314

b

Strain 12 (AAA20527)	Strain 86-028NP (AAX88733)	Strain Hi375 (AIT67726)
N636	N630	I640
N642	S636	H646
N709	N704	T715
N806	F801	F808
N912	N906	Q915
N928	I922	K931
N946	S940	D949
N952	H946	H955
N964	N958	D967

Supplementary Figure S3. Localization of *N*-glycosylated Asn residues in three different HMW1 adhesins of *NTHi*. (a) Multiple partial alignment of the HMW1 adhesins of strains 12 (AAA20527), 86-028NP (AAX88733) and 375 (AIT67726). The locations of *N*-glycosylated Asn residues were taken from a previous publication⁴. Black and red triangles point to *N*-glycosylated Asn residues conserved and not conserved, respectively, in the three aligned proteins. (b) shows the Asn residues of HMW1 of strain 12 in comparison with those of the other two strains. Conservative and non-conservative amino acid substitutions are shown in a grey and black background respectively.



Supplementary Figure S4. NTHi 54997 biofilms treated with cellulase. Cells of NTHi 54997 were distributed in the wells of a microtiter plate which was incubated for 6 h at 37 °C in a 5% CO₂ atmosphere. Grey bars indicate growth (adherent plus non-adherent bacteria). **(a)** The medium was withdrawn and cellulase (dissolved in 50 mM sodium citrate buffer, pH 4.6) added. The final volume was adjusted with distilled water to 200 µl and incubation was continued for 90 min at 37°C under 5% CO₂. A control biofilm (without enzyme) was incubated under identical conditions. Biofilm formation was quantified by staining with CV (black bars). * $P < 0.01$ and ** $P < 0.001$ compared to the control. **(b–g)** CLSM images of NTHi biofilms. NTHi 54997 biofilms were untreated (**b–d**) or treated (**e–g**) with 40 U mL⁻¹ of cellulase for 90 min at 37 °C in a 5% CO₂ atmosphere. After treatment, the cells in the biofilms were stained with the BacLight LIVE/DEAD kit to reveal viable (green fluorescence) and non-viable (red fluorescence) bacteria. In all images the scale bar = 25 µm.

Supplementary references

1. Harrison, A. *et al.* Genomic sequence of an otitis media isolate of nontypeable *Haemophilus influenzae*: comparative study with *H. influenzae* serotype d, strain KW20. *J. Bacteriol.* **187**, 4627–4636 (2005).
2. Mell, J. C. *et al.* Complete genome sequence of *Haemophilus influenzae* strain 375 from the middle ear of a pediatric patient with otitis media. *Genome Announc.* **2**, e01245-01214 (2014).
3. Frazer, K. A., Pachter, L., Poliakov, A., Rubin, E. M. & Dubchak, I. VISTA: computational tools for comparative genomics. *Nucleic Acids Res.* **32**, W273–W279 (2004).
4. Gross, J. *et al.* The *Haemophilus influenzae* HMW1 adhesin is a glycoprotein with an unusual N-linked carbohydrate modification. *J. Biol. Chem.* **283**, 26010–26015 (2008).