**Table S1. Studies addressing *H. influenzae* biofilms**

|  |  |  |  |
| --- | --- | --- | --- |
| **Strains** | **Biofilm model** | **Main results** | **Ref.** |
| **BIOFILM FORMING ABILITY OF *H. INFLUENZAE*** | | | |
| Isolates from MEF of children with OM and from sputum of adults with COPD; M37 and M37-1 (pilus deficient variant) | 96-well plate | Clinical isolates with substantial variability in their ability to form biofilms.  Cell envelopes preserved expression of P2, P5 and P6 proteins in biofilms compared to planktonic cells.  M37-1 with a marked impairment in biofilm formation compared to M37. | (Murphy & Kirkham 2002) |
| Isolates from NP and MEF of children with AOM | 96-well plate | 84.3% of the NP isolates were good biofilm forming strains.  92.3% of NTHi strains isolated simultaneously from NP and MEF had the same PFGE patterns and biofilm forming ability. | (Moriyama et al. 2009) |
| Isolates from patients with OM, NB-CAP, COPD, and invasive disease and healthy children | 96-well plate | NTHi isolates from OM and invasive disease with higher adhesion and biofilm formation ability compared with isolates from NB-CAP, COPD and healthy colonized subjects. | (Puig et al. 2014) |
| Isolates from NP of healthy children, NP of children with first AOM without conjunctivitis, NP of children with RAOM without conjunctivitis, NP of children with AOM and conjunctivitis, MEF of children with AOM treatment failure or RAOM | 96-well plate | Biofilm production in MEF samples and NP samples did not significantly differ. Presence of conjunctivitis was significantly associated with low biofilm production. | (Mizrahi et al. 2014) |
| **INFLUENCE OF SPECIFIC MOIETIES OF LIPOOLIGOSACCHARIDES (LOS) ON *H. INFLUENZAE* BIOFILMS** | | | |
| **Sialic (N-acetyl-neuraminic) acid** | | | |
| Rd KW-20; 3198; 7502; 86-0298NP; M37; LB2; LB5; mr31; 3031;  2019 wild type and *siaB* mutant | 96-well plate;  Continuous-flow system | Diminished biofilm formation for the *siaB* mutant.  Presence of sialic acid favoured bacterial growth.  LOS from NTHi growing as biofilms were sialylated. | (Swords et al. 2004) |
| 2019 wild type and *galE, lic3A, lsgB, pgm, wecA, siaA, siaB* mutants | 96-well plate;  Continuous-flow system | Diminished biofilm formation for strain 2019 with mutations in *siaB*, *siaA*, and *wecA*.  Similar or enhanced biofilm formation for the other mutants.  Sialic acid present in biofilms formed by strain 2019 and *pgm* mutant in an α2,6 linkage. | (Greiner et al. 2004) |
| 2019 wild type and *lsgB, pgm, wecA, siaA, siaB* mutants | Chinchilla model\* | Inability or markedly reduced ability to form biofilms for NTHI 2019 with mutations in *siaB, siaA, wecA*, *lsgB*, and *pgm*.  Mutants *wecA, lsgB*, and *siaA* survived in the chinchilla, inducing culture-positive MEF.  Mutants *pgm* and *siaB* extremely sensitive to chinchilla serum. | (Jurcisek et al. 2005) |
| **Phosphorylcholine (PCho)** | | | |
| 2019 wild type and *siaB* and *licD* mutants;  NTHI 86-028NP wild type and *licD* mutant | 96-well plate;  Continuous-flow system;  Permeable membrane inserts with epithelial cells | PCho content of LOS increased with biofilm growth of 2019 and 86-028NP.  PCho positive variants in the interior portion of biofilm communities on epithelial cell surfaces.  Sialylated variants distributed throughout the biofilm.  LOS from biofilms elicited less inflammatory response. | (West-Barnette et al. 2006) |
| 86-028NP wild type and *licD* mutant | Chinchilla model\* | 86-028NP *licD* mutant elicited greater inflammatory response early after challenge and induced slower progression of disease.  *licD* mutant formed less dense biofilms after 7 days.  Animals infected with the *licD* mutant had no visible biofilm after 14 days. | (Hong, Mason, et al. 2007) |
| 2019 wild type and *licD* and *licON* mutants | Continuous-flow system;  Chinchilla model\* | 2019 *licON* and *licD* mutants with increased and diminished biofilm formation ability.  2019 *licON* caused a greater proportion of biofilm-positive ears.  Animals infected with 2019 *licD* had no visible biofilms after 14 days. | (Hong, Pang, et al. 2007) |
| **CHARACTERIZATION OF *H. INFLUENZAE* BIOFILM FORMATION AND INFLUENCE OF SPECIFIC FACTORS** | | | |
| Isolates from sputum of adults with COPD and mutants deficient in expression of peroxiredoxin-glutaredoxin | 96-well plate | Peroxiredoxin-glutaredoxin present in greater abundance in biofilms.  Mutants showed a 25-50% reduction in biofilm formation ability. | (Murphy et al. 2005) |
| 9274 | Glass coverslips; Millipore filters | LOS localized in the EPS.  P6 OMP localized in the membrane of viable bacteria within the biofilm.  Hap and HWM1/HMW2 associated with bacteria within the biofilm and present in the EPS.  IgA1 protease found associated with NTHi in the biofilm and in the EPS but more concentrated in the top region of the biofilm. | (Webster et al. 2006) |
| 86-028NP | Chinchilla model\* | Biofilms contained syalilated LOS, type IV pili and dsDNA.  DNA appeared arranged in a dense interlaced meshwork of fine strands as well as in individual thicker strands crossing water channels. | (Jurcisek & Bakaletz 2007) |
| 86-028N wild type and *pilA* mutant | Continuous-flow system;  Chinchilla model\* | 86-028NP *pilA* mutant formed *in vitro* biofilms with decreased depth and density, had decreased ability to adhere to normal bronchial epithelial cells and formed less stably adherent *in vivo* biofilms with lower organized structure. | (Jurcisek et al. 2007) |
| 54997, 86-028NP, 375 *opsX* mutant, Rd KW20 | 96-well plate | 54997 and Rd KW20 were the best and worst biofilm-forming strains.  Exposure to proteolytic enzymes and DNaseI led to dispersal of biofilms.  RNase inhibited biofilm formation.  Biofilm matrix composed by β-(1→4)-glucan. | (Domenech et al. 2016) |
| 86-028NP wild type and *pilA* and *comE* mutants | 8-well glass slide; | 86-028NP formed biofilms containing fractal structures of short length scale. | (Das et al. 2017) |
| 86-028NP, 2019 wild type and *htrB, rfaD, pgmB* and *siaB* mutants | Chinchilla model\* | Biofilms containing viable bacteria and host cells within a fibrous DNA matrix.  Elastase and histone present within the biofilm close to NTHi aggregates.  2019 resistant to killing within NET structures, while *htrB, rfaD, rfaF, pgmB* and *siaB* mutants were more susceptible to NET killing. | (Hong et al. 2009) |
| **ROLE OF QS ON *H. INFLUENZAE* BIOFILMS** | | | |
| **LuxS/AI-2** | | | |
| Several clinical isolates and *luxS* mutants | Continuous-flow system | Wild type and *luxS* mutants produced identical biofilms.  *luxS* mutants had increased ability to invade eukaryotic cells.  *luxS* mutant more virulent in the chinchilla model. | (Daines et al. 2005) |
| 86-028NP wild type and *licD, licON* and *luxS* mutants | Continuous-flow system;  Chinchilla model\* | 86-028NP *luxS* mutant formed biofilms with decreased biomass, thickness, roughness and surface/volume ratio compared to 86-028NP.  *luxS* mutant had lower PCho levels than the wild type strain yet higher levels than the *licD* mutant.  After 21 days, all ears infected with 86-028NP and one ear infected with 86-028NP *luxS* contained biofilms. | (Armbruster et al. 2009) |
| 86-028NP wild type and *licD, luxS, rbsB* and *rbsB*:*luxS* mutants | Continuous-flow system;  Chambered coverglass;  Chinchilla model\* | 86-028NP *luxS* and *rbsB* mutants formed biofilms with decreased biomass, thickness and surface/volume ratio compared to the 86-028NP.  *rbsB* mutant had PCho levels similar to those of *luxS* mutant, lower than those of the wild type strain and higher than the *licD* mutant.  Bacterial persistence defect observed *in vivo* for *luxS* and *rbsB* mutants after 21 and 28 days of infection. | (Armbruster et al. 2011) |
| 86-028NP wild type and *luxS* mutant and WES204 (xylose-inducible *luxS* 86-028NP) | Continuous-flow system;  Chambered coverglass;  24-well plate;  Chinchilla model\* | Induction of *luxS* with xylose increased WES204 biofilm thickness and biomass regardless of the stage of biofilm development and the transcription of a predicted family 8 glycosyltransferease (*gstA*).  86-028NP *gstA* mutant formed biofilms with decreased thickness, biomass and extracellular matrix compared to the wild type strain.  Significant decrease in bacteria *in vivo* in the biofilm population for the *gstA* mutant*.* | (Pang et al. 2018) |
| **QseB/C** | | | |
| 3655 wild type and *luxS* and *qseC* mutants | 96-well plate;  Continuous-flow system | 3655 *qseC* mutant had decreased biofilm formation ability under static and semi-static conditions.  Total biomass of 48 h-biofilms formed in continuous flow conditions by the *qseC* mutant was reduced in relation to the wild type strain.  No differences were observed in AI-2 production capacity of 3655 and its *qseC* mutant in contrast to the *luxS* mutant. | (Ünal et al. 2012) |

MEF = middle ear fluid; NP = nasopharynx; OM = otitis media; COPD = chronic obstructive pulmonary disease; NB-CAP = nonbacteremic community-acquired pneumonia; \* experimentally infected via transbullar injection

**Table S2. Studies addressing *S. pneumoniae* biofilms**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Strains** | **Biofilm model** | | **Main results** | **Ref.** | |
| **CHARACTERIZATION OF *S. PNEUMONIAE* BIOFILM FORMATION AND INFLUENCE OF SPECIFIC FACTORS** | | | | | |
| ATCC 6303;  8 nasopharyngeal isolates;  3 external ear isolates;  1 isolate from sputum | Continuous-flow system | | Mature biofilms differed in architecture.  Proteomic changes between planktonic and 3-day old biofilms.  Proteins involved in virulence, adhesion, and resistance more abundant in biofilm cells. | (Allegrucci et al. 2006) | |
| Isolate from blood | 6-well plate | | Proteomic changes detected during biofilm development (>80%).  Proteins involved in the glycolytic pathway, translation, transcription and virulence were downregulated.  Proteins with a role in pyruvate, carbohydrate, and arginine metabolism were upregulated. | (Allan et al. 2014) | |
| R6 wild type and several mutants; M11 wild type and several mutants; ST344; 13868; D39; TIGR4; Spain6B-2; SSISP9V/1; Spain14-5; G54; SSISP23F/1; 7077/39 | 96-well plate;  Glass-bottomed dishes | | LytA amidase, LytC lysozyme, LytB glucosaminidase, CbpA adhesin, PcpA putative adhesin, and PspA surface protein mutants had decreased capacity to form biofilms.  Presence of capsule reduced biofilm development by more than 60%.  Biofilm formation was impaired in the presence of DNase I or proteases. | (Moscoso et al. 2006) | |
| ATCC 6303; Nasopharyngeal isolate | Continuous-flow system | | Small nonmucoid variant (SCV) emerged during the initial attachment stage and dominated over the course of biofilm growth.  Mucoid variants with different sizes at later biofilm developmental stages.  Reduction in colony size/mucoidy correlated with a decrease in capsule production and an increase in initial attachment. | (Allegrucci & Sauer 2007) | |
| Clinical isolate | 0.2 µm filter on blood agar plate | | Rough phase variants had diverse mutations, i.e. single nucleotide polymorphisms in the *cps3D* gene and in the putative-10 promoter and deletions in the *cps3D* gene.  Phase variants had less than 12% of the parental amount of capsule. | (McEllistrem et al. 2007) | |
| Nasopharyngeal isolate | Continuous-flow system | | Small-colony variants (SCV) dominated during initial attachment (day 1), decreasing during mature biofilm stages (days 3 to 9).  Small mucoid variants detected on 3-day-old biofilms, increasing threefold by day 9.  SCVs had decreased capsule production. | (Allegrucci & Sauer 2008) | |
| 6 nasopharyngeal isolates | 6-well plate | | Good biofilm forming strains had increased carbohydrate matrix and high antibiotic resistance.  Biofilm matrix composed of eDNA that was degraded by DNAse treatment.  Expression of *cpsA* gene downregulated in biofilm phenotype. | (Hall-Stoodley et al. 2008) | |
| M23 wild type and several mutants | 96-well plate | | Colony variants showed promoter mutations and duplications, deletions and point mutations in the *cap3A* gene.  Increased biofilm-forming ability correlated with a reduction in colony size and in the relative amount of capsular polysaccharide.  One mutant with very low capsular polysaccharide production had impaired biofilm formation. | (Domenech et al. 2009) | |
| TIGR4 wild type and *cps*4D-mutant | 96-well plate;  Continuous-flow system | | Biofilm formation by TIGR4*cps*4D- mutant (impaired capsular polysaccharide) was increased compared to the wild type strain. | (Qin et al. 2013) | |
| R6 | Glass-bottomed dishes | | Presence of eDNA, proteins and polysaccharides in the biofilm matrix detected by different methods. | (Domenech, García, et al. 2013) | |
| M11;  Several clinical isolates | 96-well plate | | Most isolates from serotypes (ST) 19A and 19F, but not 19B and 19C, as well as ST 6 formed more biofilm than the unencapsulated strain M11.  ST19A and 19F capsules contain the same type of disaccharides of ST6. | (Domenech et al. 2014) | |
| M11;  Several clinical isolates | 96-well plate | | 14 out of 16 ST were not good biofilm formers.  ST 11A and 35B formed more than 45% of the biofilm produced by M11 strain. | (Domenech et al. 2015) | |
| D39; RX1; SP670; SP456 | Glass coverslips  (24-well plate without/with substratum of  epithelial cells) | | All strains, except SP456, could be transformed in broth cultures after exogenous addition of competence-stimulating peptide (CSP).  None encapsulated isolates had natural competence during planktonic growth.  All encapsulated strains formed biofilms that integrated resistance cassettes after exogenous DNA exposure, with no addition of CSP. | (Laura R Marks et al. 2012) | |
| R704 wild type and several mutants | Glass-bottomed dishes | | In early stages of biofilm formation (4 h), *S. pneumoniae* became competent when treated with CSP, while only a small fraction of cells was transformed in 8 h-biofilms. | (Wei & Håvarstein 2012) | |
| R6;  Clinical isolate | 96-well plate | | The deposition of C3b on biofilms was impaired compared to planktonic cultures.  Binding of C-reactive protein and C1q to the bacterial surface was reduced in biofilms.  Phagocytosis of biofilms was impaired. | (Domenech, Ramos-Sevillano, et al. 2013) | |
| D39;  Clinical isolate | 24-well plate with substratum of epithelial cells | | Ten-fold more bacteria present in the supernatant than in biofilms.  Exogenous application of norepinephrine, ATP, glucose, epithelial cell lysate or temperature variation induced rapid egress of cells from biofilms to supernatant.  Dispersed bacteria had distinct phenotypic properties, with virulence genes upregulated. | (Marks et al. 2013) | |
| D39;  Clinical isolate | Glass coverslips  (24-well plates with a substratum of epithelial cells) | | Biofilm-derived fomites more tolerant to desiccation than planktonic cells and retained infectivity, being able to colonize a murine model.  Planktonic cells rapidly lost viability after inoculation on hands, while biofilm cells could be recovered at high densities after 3 h. | (Marks et al. 2014) | |
| **ROLE OF QS ON *S. PNEUMONIAE* BIOFILMS** | | | | | |
| **LuxS/AI-2** | | | | | |
| D39 wild type and *luxS* and *luxS*+ mutants | | 24- or 96-well plate | Addition of Fe(III) enhanced biofilm formation.  Fe(III) upregulated expression of *luxS*.  D39*luxS* mutant had impaired biofilm formation even in the presence of Fe(III).  D39*luxS*+ had enhanced biofilm formation capacity even without Fe(III). | | (Trappetti, Potter, et al. 2011) |
| D39 wild type and *luxS* mutants;  R6 | | 8-well glass slide;  24- or 96-well plate | *luxS* was maximally transcribed in early mid-log phase of growth.  Mutants produced 80% less biofilm biomass than D39.  Complementation or purified AI-2 restored biofilm levels.  Biofilms formed by *luxS* mutants were undetectable at early time points, in contrast of D39.  Levels of *lytA* and *ply* were regulated by *luxS.* | | (Vidal et al. 2011) |
| D39 wild type and *luxS*, *comC* and *ply* mutants;  AC2394 wild type and *ply* mutant | | 8-well glass slide;  24-well plate and snapwell filters (without/with substratum of epithelial cells) | Ply was expressed in early phases of biofilm development.  Biofilm formation by *ply* and *luxS* mutants was impaired at early time points in comparison to D39 and *comC* mutant. | | (Shak et al. 2013) |
| D39 wild type and *luxS* mutant | | 24-well plate;  8-well glass slide;  rat model | D39*luxS* mutant formed biofilms with decreased biomass, thickness and organization compared with D39.  Rat middle ears inoculated with D39 revealed dense biofilm-like cell debris deposited on the cilia, in contrast to the little cell debris in the middle ears of rats infected with *luxS* mutant. | | (Yadav et al. 2018) |
| **Com** | | | | | |
| D39; RX1 wild type and *comC* and *comD* mutants;  TIGR4; FP23 wild type and *comC* and *comD* mutants | | 6- or 96-well plate | Unencapsulated derivatives FP23 and RX1 strains formed biofilm structures comparable to TIGR4 and D39 when incubated with CSP.  TIGR4 and D39 and their *comC* negative derivative mutants were able to form biofilms upon addition of CSP.  Mutants for the CSP receptor (*comD*) did not produce any biofilm structure. | | (Oggioni et al. 2006) |
| D39;  RX1 wild type and *comC* and *comD* mutants;  TIGR4 wild type and c*ps, comC*, *comD, luxS, blpH, cps* & *comC, cps* & *comD* mutants | | 6- or 96-well plate;  Continuous flow system | TIGR4 and RX1 *cps* and *comD* mutants attachment during the first hours of incubation was independent from the addition of CSP.  Maintenance of biofilms for prolonged incubation times, for wild type and *comC* mutants, was dependent on the addition of CSP.  In continuous flow system, high values of biofilm cell counts, thickness and surface were obtained for *cps* mutant compared to the wild type and *comD* mutant. | | (Trappetti, Gualdi, et al. 2011) |
| R6D; D39; PN4595-T23 wild type and *briC* mutant | | Glass bottomed dishes | No differences in biofilm biomass and thickness at 24 h post-seeding obtained for wild type strains and *briC* mutant.  At 72h post-seeding, biofilms formed by *briC* mutant had a reduced biomass and thickness compared to the wild type.  Complementation restored biofilm phenotype. | | (Aggarwal et al. 2018) |
| **LuxS/AI-2 & Com** | | | | | |
| D39;  R6 wild type and *luxS* and *comC* mutants | | 8-well glass slide,  6- or 24-well plate and snapwell filter (with/without substratum of epithelial cells) | R6 *luxS* mutant produced biofilms with less biomass than the wild type at static and continuous flow conditions.  R6 *comC* mutant produced biofilms on abiotic surfaces with no significant differences from those formed by the wild type at early stages in static conditions.  Significant differences in biofilms formed on epithelial cells in both static and continuous flow conditions at 8 h. | | (Vidal et al. 2013) |
| **Rgg/SHP** | | | | | |
| D39 wild type and *rgg* deletion and overexpression mutants | | 24-well plate (with substratum of epithelial cells) | D39 and its *rgg* deletion mutant had no significant differences in the surface polysaccharide.  Overexpression *rgg* mutant produced an increased surface polysaccharide.  Overexpression strain formed less biofilm than the wild type strain, while the deletion mutant formed more. | | (Junges et al. 2017) |

**Table S3. Studies addressing *M. catarrhalis* biofilm formation and the influence of specific factors**

|  |  |  |  |
| --- | --- | --- | --- |
| **Strains** | **Biofilm model** | **Main results** | **Ref.** |
| O35E wild type and *uspA1* and *hag* mutants;  O35E transformant with transposon insertions in *uspA1;*  O46E wild type and *uspA1* mutant | 24-well plate | O46E readily formed biofilms, while O35E formed less biofilm.  Inactivation of *uspA1* gene caused a reduction in biofilm formation.  Hag expression correlated with impairment in biofilm formation. | (Pearson et al. 2006) |
| O35-E;  ETSU-9 wild type and *uspA1, uspA2H, uspA1&uspA2H* and *uspA2H* 418 aa deletion mutants;  ETSU-9 with transposon insertions in *uspA2H* | 24-well plate | Transposon insertion mutagenesis identified six mutants that exhibited reduced abilities to form biofilms.  Three mutants had transposon insertions in the *uspA2H* gene.  Several random insertion mutagenesis of the *uspA2H* gene adversely affected biofilm formation. | (Pearson & Hansen 2007) |
| 195 isolates from children and adults with respiratory diseases | 24-well plate | Differences in biofilm formation observed between isolates cultured from children and adults and between isolates carrying the mutually exclusive *uspA2* and *uspA2H* genes. | (Verhaegh et al. 2008) |
| 7169 wild type and *pil*AK4 mutant | Continuous flow system | *pilAk4* mutant had defects in initial attachment and exhibited delayed microcolony formation and diminished 3D expansion.  After 3 days, the mutant produced less thick biofilm compared with 7169. | (Luke et al. 2007) |
| ATCC 43617;  ATCC 2523;  ETSU-9 | Sorbarod cellulose filter-based continuous flow system | In biofilm cells, 54 genes were upregulated, while 29 were downregulated, compared to the planktonic state.  Genes that were upregulated encoded enzymes involved in nitrate reduction, and nitrite and nitric oxide reductases. | (Wang et al. 2007) |
| O35E wild type and *hfq* mutants | Sorbarod cellulose filter-based continuous flow system | Outer membrane protein profiles of O35E and the *hfq* deletion mutant showed an increased abundance of CopB, OMP G1b, and OMP J.  *hfq* mutant predominated in overnight biofilms inoculated with a 1:1 culture of wild type:mutant strains. | (Attia et al. 2008) |
| 25238 and 25239 wild type and *nucM* mutants | 48-well plate;  Calgary biofilm device | *nucM* mutants biofilms had increased biomass and more pronounced 3D structure compared with wild type strains.  Up to 1-log fewer viable cells recovered from biofilms formed by wild type strains than by *nucM* mutants. | (Tan et al. 2019) |

**Table S4. Multispecies biofilm studies involving otitis media pathogens**

|  |  |  |  |
| --- | --- | --- | --- |
| **Strains** | **Biofilm model** | **Main results** | **Ref.** |
| ***H. influenzae & S. pneumoniae*** | | | |
| 86-028NP **&** TIGR4 | Chinchilla model\*;  24-well plate | 89% *versus* 50% of the ears of mixed and single infection animals contained biofilms.  The presence of *H. influenzae* increased *S. pneumoniae* biofilm formation *in vitro*. | (Weimer et al. 2010) |
| 289, 2019, and 86-028NP **&** clinical isolates | 96-well plate with epithelial cells | Multispecies combinations increased biofilm formation compared to a single strain for most NTHi/*S. pneumoniae* combinations.  Contact with epithelial cells increased biofilm formation and stability. | (Krishnamurthy & Kyd 2014) |
| 86-028NP **&** NP isolate MNZ1113 | 24-well plate | Amoxicillin treatment completely killed MNZ1113 in monospecies biofilms but did not affect viable counts in mixed biofilms.  Co-culture with a beta-lactamase-deficient NTHi did not provide any protection against amoxicillin. | (Murrah et al. 2015) |
| Rd KW20, 86-028NP and R3157 **&** D39 wild type and *lytA*−, *spxB*−, *glpO*− mutants and OM isolate 11 | 96-well plate;  Continuous flow system | Both species formed mono and mixed biofilms in late log phase.  *S. pneumoniae* outcompeted NTHi in a stationary phase.  Survival of NTHi in co-culture was pH dependent.  Transcriptomic changes occurred for both species depending on the pH.  Changes in cell morphology and in gene expression were observed for both species. | (Tikhomirova et al. 2015) |
| 181 **&** 191 isolates from nasopharynx of children with AOM | 96-well plate | 64.6% *H. influenzae* and 66.8% S*. pneumoniae* strains produced biofilms. The proportion of biofilm-producing *H. influenzae* strains was greater with the isolation of *S. pneumoniae* in the same sample.  94.6% of cases with combined isolation showed biofilm production by *S. pneumoniae* or *H. influenzae.* | (Vermee et al. 2019) |
| ***H. influenzae* & *M. catarrhalis*** | | | |
| 86-028NP wild type and *licD, licON, luxS* and *siaB* mutants **&** 7169 | Glass slides;  Continuous flow system;  24-well plate;  Chinchilla model\* | Multispecies biofilms provided protection of NTHi to ampicillin and of *M. catarrhalis* to clarithromycin.  Protection of *M. catarrhalis* to antibiotic treatment was diminished in biofilms formed with NTHi mutants with biofilm defects (*licD*, *luxS, siaB*), and increased in biofilms formed with NTHi *licON.*  AI-2 promoted *M. catarrhalis* biofilm antibiotic resistance. Polymicrobial infection increased *M. catarrhalis* persistence *in vivo.* | (Armbruster et al. 2010) |
| ***S. pneumoniae* & *M. catarrhalis*** | | | |
| EF3030 wild type and *luxS-* mutant **&** O35E wild type and O35E*bro-* mutant | 24-well plate;  4-well glass slide;  Mouse and chinchilla model\* | Multispecies biofilms provided protection of *S. pneumoniae* to amoxicillin and of *M. catarrhalis* to azithromycin.  Antibiotic protection was also provided to *M. catarrhalis* that formed biofilms with a *S. pneumoniae luxS* mutant.  Bacterial loads were increased *in vivo* in mixed infections. | (Perez et al. 2014) |
| ***H. influenzae* & *A. otitidis*** | | | |
| ATCC 33391, OM isolates NT176 and NT1159 **&** ATCC 51267 | 96-well plate;  8-well glass slide;  Calgary biofilm device | Total biofilm biomass and viable *H. influenzae* recovered from biofilms was increased in polymicrobial infection compared to single species infection in depleted media or at suboptimal temperature.  Polymicrobial infections usually decreased antimicrobial susceptibility. | (Chan et al. 2017) |

**Table S5. Otitis media therapeutic approaches**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Strains** | **Biofilm model** | **Treatment** | **Main results** | **Ref.** |
| ***H. influenzae*** | | | | |
| Isolate from an ear infection | 96-well plate;  Continuous flow system | EDTA | Static biofilms treated with EDTA had no tower-like structures and reduced attached cell layer with a threefold biomass reduction in comparison with control.  EDTA treatment of continuous-flow biofilms resulted in thinner and less structured biofilms with a 42% biomass reduction. Combination of EDTA or DNaseI with ampicillin or ciprofloxacin had a synergistic effect on biofilm biomass reduction. | (Cavaliere et al. 2014) |
| 86-028NP | 8-well glass slide; Chinchilla model\* | Polyclonal antibodies against IHF | *In vitro* biofilm height, biomass, and thickness were diminished by more than 80 % upon incubation with anti-IHF.  Biofilm treatment with anti-IHF combined with amoxicillin was synergistic, with an increased reduction in height and bacterial dead. | (Goodman et al. 2011) |
| 86-028NP | 8-well glass slide;  Transwell into 96-well plate | Polyclonal antibodies against IHF | Anti-IHF tethered to agarose beads had similar effect on biofilm reduction than direct application of anti-IHF.  Combination of anti-IHF with antibiotics facilitated biofilm disruption and killing of adherent and planktonic bacteria at concentrations equal to 8-fold lower than MIC90. | (Brockson et al. 2014) |
| 86-028NP;  1714;  1885 | 8-well glass slide;  Chinchilla model\* | Monoclonal antibodies against IHF | Treatment of biofilms with either of two tip-directed MAbs reduced biomass six to seven-fold.  Administration of both in a cocktail had an additive effect.  There was little to no evidence of a mucosal biofilm as well as of inflammation remaining within the middle ears of animals treated with MAbs alone or in combination. | (Novotny et al. 2016) |
| 86-028NP | Chinchilla model\* | NTHI OMP P5- and TFP-targeted immunogens | Immunization with the OMP P5- and TFP-targeted immunogens alone or with an adjuvant reduced biofilm biomass within the middle ears and the number of ears having middle ear fluid. | (Novotny et al. 2011) |
| 86-028NP | Chinchilla model\* | NTHI OMP P5- and TFP-targeted immunogens | Immunization with a band-air vaccine with a chimeric antigen targeting OMP P5 and TFP and an adjuvant in the postauricular region reduced NTHI levels by 3- and 2-log-units in MEF and by 3- and 1-log-unit in mucosal biofilms, compared to saline and adjuvant alone. | (Novotny, Clements, et al. 2015) |
| 86-028NP wild type and *pilA* and *luxS* mutants | 8-well glass slide | Antibodies against pilA (major subunit of TFP) | Treatment with anti-rsPilA reduced biofilms biomass by 82% compared with naive serum.  Minimal residual biomass that maintained traditional biofilm structure.  Incubation of biofilms formed by *pilA* and *luxS* mutants with anti-rsPilA did not induce significant changes in structure or biomass. | (Novotny, Jurcisek, et al. 2015) |
| ATCC 10211;  11 clinical isolates | 96-well plate; | N-ethyl-3-amino-5-oxo-4-phenyl-  2,5-dihydro-1H-pyrazole-1-carbothioamide | The pyrazol derivative showed an inhibitory effect against biofilm-forming cells of *H. influenzae* ATCC 10211 (MBIC = 15.63 µg ml-1) or 7 *H. influenzae* clinical isolates (MBIC = 0.49–31.25 µg ml-1). In 4 isolates, MBIC were found to be higher than 31.25 µg ml-1. | (Kosikowska et al. 2014) |
| 3 clinical isolates | 96-well plate; | Natural and synthetic chalcones | Natural chalcones 1 and 2 inhibited biofilm formation by NTHi, and were more potent inhibitors than azithromycin.  Synthetic chalcone 8 (3-hydroxychalcone) was most effective, showing concentration-dependent inhibitory effects. | (Kunthalert et al. 2014) |
| ATCC 49247; 20 clinical isolates | 96-well plate; | *Oldenlandia diffusa* Extract (OdiE) | Biofilm formation was reduced in the presence of OdiE in a concentration-dependent manner.  mRNA level of *luxS* was reduced soon after OdiE addition, in contrast with that of *qseC* that did not change. | (Wajima et al. 2016) |
| Isolate from sputum | 6-well plate;  Glass bottomed dishes;  transwell with epithelial cells | Cephalosporin-3-diazeniumdiolate nitric oxide (NO) donor  Prodrug (PYRRO-C3D) | Combined treatment of PYRRO-C3D with azithromycin resulted in a significant increase in bacterial killing (1 log fold reduction in biofilm viable cells and biomass) compared with each treatment alone.  In biofilms grown on respiratory epithelia, the combination was also synergistic (2-log-unit reduction of viable cells). | (Collins et al. 2016) |
| Isolate from sputum | 24-well plate;  Glass coverslips | D-methionine | Growth of biofilms with 20 mM of exogenous D-methionine reduced viable cells by 1 log-unit.  Compact aggregation of cells and evidence of abnormal cellular morphology.  124 proteins were differentially expressed, with 9 proteins involved in peptidoglycan synthesis and cell division showing increased expression. | (Dawe et al. 2017) |
| NP isolates | 6-well plate; | SurgihoneyRO | SurgihoneyRO reduced biofilm viable cells in a concentration dependent manner, with 213 g/L reducing 5-log units.  Dose-dependent increase in H2O2 levels in the media observed. | (Newby et al. 2018) |
| ***S. pneumoniae*** | | | | |
| P046 (*lytA lytC* mutant) | 96-well plate;  glass-bottomed dishes | LytA, LytC, Pal, Cpl-1,  Cpl-7, and Ejl | LytA and the LytA-like Ejl produced the most noticeable disintegration (around 80%) of biofilms, followed by Cpl-7 (70%) and Cpl-1 (55%). Pal had no apparent effect on biofilm disintegration but killed nearly 90% of viable cells. | (Domenech et al. 2011) |
| 20 isolates from MEFof children | 96-well plate | Xylitol | Growth of biofilms in media containing xylitol resulted in lower OD values compared with the control.  In the presence of glucose or fructose, biofilm formation was enhanced, and the inhibitory effect of xylitol was not observed. Xylitol decreased *lytA* expression levels. | (Kurola et al. 2011) |
| D39;  SP670; EF3030;  JY53 | Glass coverslips  (24-well plates with a substratum of epithelial cells) | Human Milk Protein-Lipid Complex HAMLET | Treatment of biofilms with HAMLET and penicillin was synergistic, reducing 5.2 log-units of D39 cells.  Synergism more pronounced in penicillin-resistant strain SP670.  Combination of erythromycin with HAMLET also synergistic, with near eradication of D39 biofilm biomass.  Treatment of EF3030 biofilms with HAMLET and gentamicin was synergistic, with near eradication of adherent bacteria. | (Laura R. Marks et al. 2012) |
| D39 | 96-well plate; | *Eugenia caryophyllata* (Ec) extract and eugenol | Both Ec extract and eugenol had inhibitory effect on biofilm formation.  Biofilm viable cells decreased at a faster rate than biofilm biomass. | (Yadav et al. 2013) |
| D39;  TIGR4;  SPJV01 | 8-well glass slide; 24-well plate;  Continuous-flow system | *Rubus ulmifolius* extract 220D-F2 | 220D-F2 inhibited biofilm formation in a dose-dependent manner. Treatment of biofilms with 220D-F2 significantly reduced biofilm biomass. | (Talekar et al. 2014) |
| ATCC 49619;  5 clinical isolates | 96-well plate; | *Shin’iseihaito* extract (SSHT) | SSHT significantly inhibited biofilm formation in dose- and time-dependent manners.  No differences obtained in day 1, but a significant inhibitory effect was seen on days 2 and 3. | (Minami et al. 2017) |
| R6;  P103 | 96-well plate;  glass-bottomed dishes | Ceragenin CSA-13 | CSA-13 effectively disintegrated biofilms produced by R6 strain.  No apparent disintegrating effect seen on P103 biofilms, although more than 95% of viable cells were killed. | (Moscoso et al. 2014) |
| D39;  3 clinical isolates | 6-well plate; glass  bottomed dishes; adenoid tissue | Sodium nitroprusside dihydrate (SNP) | Treatment of biofilms with SNP significantly reduced biofilm biomass and 3-log-units of viable cells.  No significant change in biofilm ultrastructure observed. Combination of amoxicillin-clavulanic acid with SNP resulted in a significant reduction in biofilm viable cells compared with antibiotic alone.  Combined treatment reduced viable cells on *ex vivo* adenoid tissue by nearly 3 log-units. | (Allan et al. 2016) |
| D39;  1 clinical isolate | 6-well plate; | Cephalosporin-3-diazeniumdiolate nitric oxide (NO) donor  Prodrug (PYRRO-C3D) | PYRRO-C3D treatment reduced biofilm viable cells in a concentration dependent manner (3-log reduction).  No change in maximum biofilm height or total biomass observed. PYRRO-C3D had similar potency to amoxicillin and greater efficacy than azithromycin. | (Allan et al. 2017) |
| MTCC 2672 | Glass coverslips | Zinc oxide (ZnO) nanoparticles | Sub-MIC concentrations of ZnO nanoparticles had anti-biofilm properties.  Reduction of colonization was maximal and minimal with concentrations of 12 and 3 µg/mL of nanoparticles. | (Bhattacharyya et al. 2018) |
| D39 | 96-well plate; | Quercetin | Biofilm formation was reduced with increasing quercetin concentrations (>12.5 μM).  The inhibitory effect of quercetin on biofilm growth was reduced in the presence of sialic acid. | (Wang et al. 2018) |
| D39;  ATCC 6303;  ATCC 49619;  7101975 clinical isolate | 24- or 96-well plate;  Rat model | Human amniotic membrane extract and chorionic membrane  extract (AME/CME) | Sub-MIC concentrations of AME/CME disabled cells of forming robust biofilms and biomass decreased more than 50%.  Eradication achieved with 4xMIC.  In rats treated with AME no biofilm-like structures were visible and less viable bacteria were recovered in contrast to the control. | (Yadav et al. 2017) |
| R6 | 24- or 96-well plate; | 5-azacytidine | Addition of 100 and 500 µM 5-aza to the growth medium significantly reduced biofilm biomass by 54 and 70%, respectively.  The inhibitory effect of 100 µM 5-aza was more significant on biofilms than on planktonic cells, but it was not able to eradicate already established biofilms.  Genes involved in the methionine and homocysteine recycling pathway (*luxS, metK, pfs* and *cmK*) were downregulated. | (Yadav et al. 2012) |
| D39 | 24- or 96-well plate | Sinefungin | Biofilms grown with 10 and 50 𝜇g/mL sinefungin had a significant decrease in biomass by 15% and 53%, respectively.  Treatment of pre-established biofilms with 50 𝜇g/mL sinefungin significantly reduced biomass and viable counts.  Decreased gene expressions of *luxS, pfs,* and *speE* was detected in biofilms grown with sinefungin compared to control. Sinefugin decreased AI-2 levels. | (Yadav et al. 2014) |
| D39;  ATCC 6303;  ATCC 49619;  7101975 clinical isolate | 24- or 96-well plate | Pyrimidinedione | Addition of 1 μM/mL pyrimidinedione significantly decreased D39 biofilm biomass by 54% and viable cells by 83% in comparison to control. Pyrimidinedione has no effect on pre-established biofilms.  Expression of 56 genes was up-regulated, while expression of 204 genes (including *comC*) was down-regulated in pyrimidinedione-grown biofilms, as compared to controls. | (Yadav et al. 2015) |
| ***M. catarrhalis*** | | | | |
| 5 clinical isolates | 96-well plate | Hyaluronic acid (HA) | 100% HA reduced about 30% of *M. catarrhalis* biofilms | (Drago et al. 2014) |
| 3 clinical isolates | Glass coupons | Antibacterial photodynamic therapy (aPDT) | aPDT decreased viable bacteria in overnight biofilms by 3–4 log-units. Biofilm cells exposed to aPDT had prominent morphological changes compared to controls. | (Luke-Marshall et al. 2014) |
| ***H. influenzae* & *S. pneumoniae*** | | | | |
| 54997 **&**  P233 | 96-well plate; | N-acetyl-L-cysteine (NAC) and  cysteamine | Exposure of mixed biofilms to 0.5 mg/ml NAC killed 99% of *S. pneumoniae* cells and practically eliminated NTHi. At 2.5 mg/ml (MIC), bacteria were virtually eradicated.  Cysteamine caused 90% reduction in the viability of both pathogens when used at 0.5 and 2.5 mg/ml.  A concentration of 5 mg/ml led to the almost total killing of NTHi and to the survival of 2% of *S. pneumoniae* cells. | (Domenech & García 2016) |
| 54997 **&**  R6 and P181 | 96-well plate; glass-bottomed dishes | Esters of bicyclic amines (EBAs) | EBA 31 at 55 μM was sufficient to completely inhibit *S. pneumoniae* biofilm formation, whereas NTHi cell viability reduced more than 90%. Incubation of preformed mixed biofilms with 220 μM of EBA31 caused reductions in the viability of 2 and 3 log-units for NTHi and *S. pneumoniae,* respectively, whereas at 550 μM almost eradicated the bacterial population. | (Roig-Molina et al. 2019) |
| ***H. influenzae* & *M. catarrhalis*** | | | | |
| 86-028NP wild type and *luxS* mutant  **&** 7169 | 8-well glass slides | Antibodies against pilA (major subunit of TFP) and OMP P5 | Treatment with anti-rsPilA serum and anti-OMP P5 reduced mixed biofilms biomass and mean thickness at two temperatures.  Biofilms exposed to a chimeric antigen targeting both OMP P5 and TFP and an adjuvant were the most inhibited.  There was no significant effect on *M. catarrhalis* mono-biofilms by any of the immunogens.  Treatment with anti-rsPilA and the chimeric antigen reduced biomass and mean thickness of pre-established biofilms, but anti-OMP P5 had no effect.  *M. catarrhalis* was dispersed from mixed biofilms formed with NTHi wild type and *luxS* complemented strain, but not from mixed biofilms formed with NTHi *luxS* mutant. | (Mokrzan et al. 2018) |