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Biofilm Formation Avoids Complement Immunity and Phagocytosis of *Streptococcus pneumoniae*

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Running title: Biofilms avoid immunity to pneumococcus

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30   *Streptococcus pneumoniae* is a frequent member of the microbiota of the human

31   nasopharynx. Colonization of the nasopharyngeal tract is a first and necessary step

32   in the infectious process and often involves the formation of sessile microbial

33   communities by this human pathogen. The ability to grow and persist as biofilms is

34   an advantage for many microorganisms because biofilm-grown bacteria show a

35   reduced susceptibility to antimicrobial agents and hinder the recognition by the

36   immune system. Host protection against biofilm-related pneumococcal disease has

37   not been defined yet. Using pneumococcal strains growing as planktonic cultures

38   or as biofilms, we have investigated the recognition of *S. pneumoniae* by the

39   complement system and its interactions with human neutrophils. Deposition of

40   C3b, the key complement component, was impaired on *S. pneumoniae* biofilms. In

41   addition, binding of C-reactive protein and the complement component C1q to the

42   pneumococcal surface was reduced in biofilm-growing bacteria demonstrating that

43   pneumococcal biofilms avoid the activation of the classical complement pathway.

44   Besides, recruitment of factor H, the down-regulator of the alternative pathway

45   was enhanced by *S. pneumoniae* growing as biofilms. Our results also show that

46   biofilm formation diverts the alternative complement pathway activation by a

47   PspC-mediated mechanism. Furthermore, phagocytosis of pneumococcal biofilms

48   was also impaired. The present study confirms that biofilm formation in *S.*

49   *pneumoniae* is an efficient way for host immune evasion both from the classical

50   and the PspC-dependent alternative complement pathways.

51

52 *S*treptococcus pneumoniae, the pneumococcus, is the leading pathogen producing

53 acute otitis media, community-acquired pneumonia, and invasive diseases

54 including bacterial meningitis and sepsis (1). The growth and dispersal of microbes,

55 whether pathogenic or environmental, commonly involve the production of biofilms,

56 which represent the primary mode of pneumococcal growth during colonization,

57 recurrent otitis media and the early stages of invasive disease (2-4). These evidences

58 support the importance of studying pneumococcal sessile communities to understand

59 key events in the pathogenesis development of this important human pathogen.

60 Biofilm formation is a complex process initiated by the attachment of

61 microorganisms to a surface or interface that is embedded in an extracellular matrix

62 constituted by various polymeric substances (5, 6). The biological and physicochemical

63 characteristics of biofilm structure protect the bacterium from environmental adversities

64 and confer the microorganism an inherent resistance to antimicrobial therapies and the

65 host immune response (6, 7). It is well known that the complement system represents

66 one of the first lines of defense against invading pathogens such as *S. pneumoniae* and

67 plays a vital role in both innate and acquired immunity (8). This unique host defense

68 mechanism is activated by three different pathways —known as the classical, alternative

69 and lectin pathways— that converge at the central component C3, which is involved in

70 essential phases of the immune response such as recognition and clearance of

71 microorganisms, inflammatory response and induction of phagocytosis (8, 9). The

72 classical complement pathway is activated by the recognition of antigen-antibody

73 complexes on the bacterial surface by the complement component C1q and it is

74 generally considered to be an effector of the acquired immune response. This cascade

75 plays a vital role for complement activation against pneumococci (10, 11). However, the

76 classical pathway has also an important role as part of the innate immune response to *S.*

77 *pneumoniae* since it is activated by other innate immune mediators such as the natural

78 IgM, the C-reactive protein (CRP), the serum amyloid P protein (SAP), or the lectin

79 receptor SIGN-R1 (10-12). Besides, the alternative pathway is activated by the

80 spontaneous hydrolysis of the C3 component, triggering the amplification of C3

81 deposition and, therefore, contributes significantly to innate immunity (13). In addition,

82 an MBL-independent lectin pathway activation has been recently demonstrated,

83 confirming the importance of complement-mediated immunity against *S. pneumoniae*

84 (14).

85   Although avoidance of complement immunity and phagocytosis is a clear advantage

86 for bacterial dissemination it may also be a common immune evasion strategy used by

87 selected pathogens to allow long-term colonization and persistent carriage. In this sense,

88 there are several studies reporting the importance of biofilm formation by various

89 microorganisms in the evasion of the host immune response (15-17), although the

90 interactions of pneumococcal biofilms with complement immunity and phagocytic cells

91 is largely unknown.

92   In this study, we have investigated the recognition by the complement system and

93 human neutrophils of *S. pneumoniae* growing either as biofilms or planktonic cultures

94 by exploring how acute phase proteins and complement down-regulators interact with

95 these two different bacterial life styles.

96 **MATERIALS AND METHODS**

97 **Bacterial strains and growth conditions.** *S. pneumoniae* non-encapsulated strains used

98 for this study were: strain R6 (a D39 derivative) (18), strain P040 expressing the green

99 fluorescent protein (GFP) [R6 (pMV158GFP) obtained by transformation with plasmid

100 DNA; tetracycline resistant], strain P064 [R6 but *pspC*::*aad9* constructed by mariner

101 mutagenesis, displaying anti-transcribed orientation of the antibiotic resistance cassette

102 of the minitransposon with respect to the targeted gene; spectinomycin-resistant] (19).

103 An encapsulated pneumococcal clinical isolate of serotype 19A (strain 1041) from a

104 patient with sepsis was used. Bacterial strains were grown at 37°C in C medium (20)

105 containing 33 mM potassium phosphate buffer at pH 8.0 (CpH8 medium) either

106 supplemented (or not) with 0.8 mg ml^{-1} yeast extract (C+Y medium). Biofilm formation

107 by pneumococcal cells was obtained using 96-well polystyrene microtiter plates (Costar

108 3595; Corning) as previously described (19, 21). Briefly, cells were grown in C+Y

medium to an optical density of 0.5–0.6 at 550 nm (OD_{550}), sedimented by

centrifugation, resuspended in an equal volume of CpH8 medium, diluted 1/100, and

then 200 µl containing 5×10^6 CFU ml^{-1} were dispensed in 96 well plates for biofilm

formation or in sterile Falcon tubes for planktonic replication growth. Both cultures

were incubated during 5 h at 34°C.

Complement factors binding to *S. pneumoniae* strains. Human serum from five

healthy male volunteers unvaccinated against *S. pneumoniae* (median age of 40 years)

were obtained with informed consent according to institutional guidelines and stored as

single-use aliquots at −70°C as a source of complement and serum components. C1q,

C3b, factor H (FH), C4b-binding protein (C4BP) and CRP were assessed using flow

cytometry assays as previously described (11). After the incubation process at 34°C,

bacterial cultures growing as biofilms in microtiter plates or in the planktonic form were

washed with fresh CpH8 medium and resuspended in phosphate-buffered saline (pH

7.0) (PBS). Biofilm disaggregation was performed by gently pipetting and slow

vortexing before the opsonization process to avoid possible bias by morphological

differences between the two growing stages. The corresponding bacterial suspensions

(20 µl) were added to tubes containing 20 µl of human serum diluted 1/5 in PBS and

samples were incubated during 20 min at 37°C to allow opsonization by the different

serum components. Previously, the number of biofilm-forming CFU was determined by

viable counts of bacteria and a similar number of planktonic cells were used in each

assay.

C1q and C3b deposition were detected by incubating the bacteria with 50 µl of a

fluorescein isothiocyanate- (FITC-) conjugated polyclonal sheep anti-human C1q

antibody (Serotec) or a FITC-conjugated polyclonal goat anti-human C3b antibody

(ICN-Cappel) diluted 1/300 or 1/500 in PBS/0.1% Tween 20 respectively. Bacterial

suspensions and antibodies were incubated during 2 h at 37°C for C1q detection or

during 30 min on ice for C3b analysis. The deposition of CRP, FH and C4BP was

investigated by using a polyclonal rabbit anti-human CRP antibody (Calbiochem), a

polyclonal sheep anti-human FH antibody (Serotec) and a polyclonal sheep anti-human

138 C4BP antibody (Serotec) for 1 h at 37°C respectively followed by a secondary staining

139 in PBS/0.1% Tween 20 containing FITC-conjugated polyclonal goat anti-rabbit, or

140 FITC/DYLIGHT 649 donkey anti-sheep antibodies (Serotec). After incubations, the

141 bacteria were washed with PBS/0.1% Tween 20 to remove unbound components, fixed

142 in 3% paraformaldehyde and analyzed on a FACS Calibur flow cytometer (BD

143 Biosciences) using forward and side scatter parameters to gate on at least 25,000

144 bacteria. The results were expressed as a relative % fluorescence index (FI) that

145 measures not only the proportion of fluorescent bacteria positive for the host serum

146 component investigated but also the intensity of fluorescence that quantify the immune

147 component bound (11, 22).

148 **C3b analysis by confocal laser scanning microscopy.** To determine C3b deposition

149 on *S. pneumoniae*, the R6 strain was first grown as biofilm and planktonic culture as

150 described above using glass-bottom dishes (WillCo-dish; WillCo Wells B. V., The

151 Netherlands) or sterile Falcon tubes, respectively. Pneumococcal biofilm and planktonic

152 culture samples were incubated with human serum as a source of complement and the

153 different samples were incubated during 20 min at 37°C to allow opsonization by the

154 C3b component. A FITC-conjugated polyclonal goat anti-human C3b antibody (ICN-

155 Cappel) was used to detect the C3b bound using a Leica TCS-SP5-AOBS-UV confocal

156 laser scanning microscope (CLSM) equipped with an argon ion laser and a Leica

157 DM4000B fluorescence microscopy. Pneumococcal cells were labeled with SYTO 59.

158 The planktonic cells were sedimented by centrifugation to improve the visualization the

159 C3b component by fluorescence microscopy. The excitation/emission maxima were

160 around 495/519 and 622/645 nm for anti-human C3b-FITC and SYTO 59, respectively,

161 and the magnification was ×100. Images were analyzed using the Leica software LCS.

162 Projections through the *x-y* plane (individual scans at 0.5-μm intervals) and the *x-z*

163 plane (images at 3-μm intervals) were obtained by CLSM.

164 **Quantification of IgG, phosphorylcholine (PCho) and PspC:** Detection of IgG,

165 Pcho or PspC on the bacterial surface of biofilms and planktonic cultures was

166 determined by flow cytometry. Briefly, the experimental conditions of the assay were

167 the same than explained above except that bacterial strains were incubated during 20

168 minutes at 37°C with 50% human serum as a source of IgG, or during 1 h at 37°C with

169 antibodies TEPC-15 (monoclonal antibody to PCho, Sigma-Aldrich) diluted 1/25 or

170 rabbit polyclonal antibody to PspC diluted 1/300 (a kind gift from Prof Sven

171 Hammerschmidt, University of Greifswald, Germany). The secondary antibodies used

172 were rabbit anti-mouse FITC (Serotec) or goat anti-rabbit FITC (Serotec) for the

173 detection of PCho or PspC respectively. Detection of IgG by flow cytometry was

174 measured after incubation for 20 minutes on ice with a Phycoerithryn- (PE) -conjugated

175 donkey anti-human IgG antibody (Jackson Immunoresearch). All secondary antibodies

176 were diluted 1/200 in PBS-Tween 20 (0.1%) and incubated for 30 minutes on ice.

177 **Phagocytosis of *S. pneumoniae* biofilms and planktonic cultures.** Experiments

178 investigating human neutrophil phagocytosis were performed by a flow cytometry assay

179 using HL-60 cells (CCL-240; ATCC) differentiated to granulocytes (11, 23). The assay

180 included the fluorescent pneumococcal P040 strain grown as biofilm or planktonic

181 culture in CpH8 medium containing 1% maltose and 1 µg ml^{-1} tetracycline (19).

182 Briefly, 96-well plates containing 1×10^6 CFU per well were infected in triplicate at a

183 ratio of 10 bacteria:1 cell with 20 µl of a suspension of the pneumococcal P040 strain

184 previously opsonized for 20 minutes with HBSS, heat inactivated serum (HIS) or the

185 human serum and the mixture containing cells was incubated for 30 minutes at 37°C

186 with shaking (150 rpm). A minimum of 6,000 cells were analyzed using a Cytomics

187 FC500 Beckman Coulter flow cytometer equipped with a 488 nm Ar-ion laser. The

188 presence of complement receptors on HL-60 granulocytes has been previously

189 documented and therefore expression of CD11b (iC3b receptor and CR3 α-chain), a

190 marker of granulocytic differentiation, was measured prior to phagocytic assays to

191 confirm the presence of the receptor (24). Results were expressed as a FI (see above)

192 defined as the proportion of positive cells for fluorescent bacteria multiplied by the

193 geometric mean of fluorescence intensity which correlates with the amount of bacteria

194 phagocytosed per cell (11, 23).

195   **Statistical analysis.** Data are representative of results obtained from repeated

196   independent experiments, and each data point represents the mean and standard

197   deviations (SD) for 3 to 5 replicates. Statistical analysis was performed by using two-

198   tailed Student´s *t* test (for two groups). GraphPad InStat version 3.0 (GraphPad

199   Software, San Diego, CA) was used for statistical analysis.

200   **RESULTS**

201   **C3b deposition on *S. pneumoniae* growing as biofilms or planktonic cultures.** The

202   deposition of the complement component C3b on the surface of *S. pneumoniae* was

203   investigated by a flow cytometry assay using bacteria grown either as biofilms or as

204   planktonic cultures. Since non-encapsulated pneumococci show a higher capacity to

205   form *in vitro* biofilms than encapsulated isolates (reviewed in reference (6)) and to

206   prevent any possible hindrance of the capsular polysaccharide on complement activity

207   (12), the non-encapsulated pneumococcal R6 strain was used. In addition, to avoid

208   possible bias in complement interaction with *S. pneumoniae* between the two modes of

209   bacterial growth, biofilm disaggregation was performed before opsonization with

210   human serum. The morphologies of *S. pneumoniae* cells from planktonic cultures or

211   from disaggregated biofilms (mainly, diplococci) were indistinguishable by phase

212   contrast microscopy confirming that disaggregation does not induce morphological

213   changes that could affect complement interaction in further assays (not shown).

214   Recognition of *S. pneumoniae* by the key complement component C3b was explored by

215   flow cytometry using a pneumococcal strain without capsule (Figs. 1A and B) and an

216   encapsulated clinical isolate of serotype 19A (Figs. 1C and D). C3b deposition on

217   pneumococcal biofilms was markedly impaired in comparison to planktonic cultures

218   suggesting that biofilm formation in *S. pneumoniae* is a mechanism used by the

219   bacterium to avoid the recognition by this key complement component (Fig. 1). C3b

220   binding was further investigated on planktonic cultures and intact biofilms of the R6

221   strain using fluorescence microscopy and CLSM, respectively (Fig. 2). C3b bound on

222   the bacterial surface was detected by using FITC-conjugated polyclonal goat anti-

223 human C3b antibody (green fluorescence), whereas the pneumococcal cells were

224 stained with SYTO 59 (red fluorescence). The entire bacterial surface of the planktonic

225 culture was coated by C3b (Figs. 2A and B), whereas only small patches of the

226 pneumococcal biofilm appear to contain C3b (Figs. 2C and D). This confirmed that

227 when *S. pneumoniae* cells form biofilms a notable reduction of the opsonization process

228 by C3b occurs.

229 **Reduced activation of the classical complement pathway by pneumococcal**

230 **biofilms.** As the classical pathway is essential for complement activation against *S.*

231 *pneumoniae* (see above), deposition of its first component, C1q, on the R6 strain was

232 investigated by flow cytometry. C1q deposition was significantly reduced on the surface

233 of pneumococcal biofilms compared to planktonic cultures (Figs. 3A and B), indicating

234 that pneumococcal biofilms hinder the activation of the classical pathway. Since

235 recognition of *S. pneumoniae* by the pentraxin CRP (an acute phase protein) increases

236 the deposition of C1q on the pneumococcal surface activating therefore the classical

237 pathway (12) we tested whether the reduced C1q level on the surface of pneumococcal

238 biofilms was somewhat mediated by an impaired binding by human CRP on biofilms.

239 Actually, binding to human CRP was strongly reduced on the surface of *S. pneumoniae*

240 R6 biofilms in comparison to planktonic cultures (Figs. 3C and D). These results taken

241 together demonstrate that pneumococcal biofilms enhance the resistance of *S.*

242 *pneumoniae* to complement immunity by diminishing the classical pathway activation.

243 Additional experiments confirmed that the impaired recognition of *S. pneumoniae*

244 biofilms by C1q and CRP was not related to differences in binding to IgG (Figs. 3E and

245 F) or variations in the amount of the PCho epitope on the bacterial surface (Figs. 3G and

246 H).

247 **Recruitment of human complement regulators.** Interaction of pneumococcal

248 cultures grown as biofilms or planktonic cultures with the major fluid-phase regulators

249 of either the classical/lectin (C4BP) or alternative (FH) complement cascades was

250 investigated by flow cytometry (Fig. 4). Deposition of C4BP was very similar in

251 planktonic and biofilm cultures indicating that interaction with the main down-regulator

252 of the classical pathway is not affected by biofilm formation (Figs. 4A and B). In

253 contrast, recruitment of FH was significantly enhanced on pneumococcal biofilms

254 compared to planktonic cultures (Figs. 4C and D), which suggested that the impairment

255 of the alternative pathway in pneumococcal biofilms is mediated by an increased

256 binding to FH, the down-regulator of the alternative cascade.

257 **PspC is involved in the enhanced resistance of pneumococcal biofilms to**

258 **complement-mediated immunity.** It has been documented that the pneumococcal

259 surface protein PspC (also designated CbpA) is able to bind FH (25-27). To explore the

260 possible involvement of PspC in the increased recruitment of FH on pneumococcal

261 biofilms, an isogenic *pspC* mutant of the R6 strain (P064 strain) was employed. Indeed,

262 loss of PspC expression in P064 cells growing as biofilms caused a decrease on FH

263 binding to levels similar to those shown by planktonic cultures of the same strain (Figs.

264 4E, F, and G). C3b deposition assays using biofilms of the wild-type and *pspC* deficient

265 strain were performed demonstrating that the increased recruitment of FH mediated by

266 PspC confers an advantage to *S. pneumoniae* growing as biofilms to avoid the

267 recognition by C3b (Figs. 5A and B). However, in the absence of PspC in both biofilms

268 and planktonic cultures, a similar C3b deposition pattern was found confirming that this

269 protein is clearly involved in the enhanced resistance of pneumococcal biofilms to the

270 complement system (Figs. 5C and D). To explore the possibility that *S. pneumoniae*

271 growing as biofilms might display greater levels of PspC to avoid complement mediated

272 immunity, detection of the PspC exposed on the bacterial surface was analyzed in

273 pneumococcal biofilms and planktonic cultures (Figs. 5E and F). Our results showed

274 increased levels of PspC on the surface of the biofilm confirming that *S. pneumoniae*

275 adopting a sessile life divert the amplification of the alternative pathway and

276 consequently the deposition of C3b by inducing higher levels of PspC on the bacterial

277 envelope.

278 **Phagocytosis by neutrophils is impaired in *S. pneumoniae* biofilms.** Activation of

279 complement immunity is a very efficient mechanism of the host immune response

280 involved in phagocytosis of pneumococci and other encapsulated bacteria. The

281 susceptibility of pneumococcal biofilms to the opsonophagocytosis mediated by human

282 neutrophils was investigated by flow cytometry using strain P040. Uptake of *S.*

283 *pneumoniae* grown as a biofilm was markedly impaired in comparison to the planktonic

284 culture demonstrating that the sessile growth of *S. pneumoniae* represents a benefit to

285 the microorganism by avoiding very efficiently the phagocytosis mediated by human

286 neutrophils (Fig. 6). However, in the absence of complement, (HBSS or heat inactivated

287 serum) phagocytosis levels were drastically reduced in comparison to bacteria

288 opsonized with serum confirming that the increased resistance to phagocytosis by

289 pneumococcal biofilms is complement dependent (Fig. 6). Overall, these findings

290 mirror the results obtained above regarding the interaction with different components of

291 the complement immune response and strongly suggest that the reduced complement

292 activation on the surface of pneumococcal biofilms confers the bacterium an enhanced

293 resistance to be phagocytosed by professional phagocytes.

294 **DISCUSSION**

295 Bacterial biofilms are widely accepted as a frequent cause of chronic persistent

296 infections (5). The ability of respiratory pathogens to persist in the nasopharynx and

297 disseminate throughout the host under certain favorable circumstances, is associated

298 with their biofilm-forming capacity on the mucosal epithelium (7). Nasopharyngeal

299 colonization provides a stable environment to *S. pneumoniae* from which it can spread

300 to other hosts and/or give rise to an infection (28). Compared to their planktonic

301 counterparts, bacteria living as biofilms appear to have developed an evolutionary

302 advantage because they are less sensitive to antibiotics, which complicates the

303 effectiveness of the antimicrobial therapy (7, 29). Two main questions that remain

304 unanswered to date is how the host defense immune system reacts to *S. pneumoniae*

305 biofilms and whether or not pneumococcal biofilms can be efficiently recognized by

306 professional phagocytes. One of the major immunological mechanisms against

307 microbial pathogens is complement-mediated immunity that consists of a complex

308 network of circulating and cell surface-bound proteins that play an essential role in host

defense (8, 9). In this sense, it has been shown that biofilm formation by *Mycoplasma pulmonis* protects from the lytic effects of complement immunity (16) whereas *Staphylococcus epidermidis* growing as biofilms have developed the possibility of avoiding neutrophil killing by preventing C3b opsonization (30). In this study, we have investigated the interaction of *S. pneumoniae* with the complement system exploring the activation and regulation of complement immunity on biofilm and planktonic bacteria. Our results indicate that pneumococcal cells within biofilms are much more effective in diverting C3b deposition on the bacterial surface than planktonic bacteria. This is relevant from the immunological perspective because C3b is essential for both the innate and adaptive immunity against pyogenic bacteria such as *S. pneumoniae* (8, 10, 11).

To unravel the mechanism behind the impaired C3b deposition on pneumococcal biofilms, the classical pathway activation was investigated, as this cascade is essential for complement immunity against pneumococcus (10, 11). Pneumococcal biofilms have been identified in children with acute otitis media (2) and therefore, the impaired classical pathway activation on *S. pneumoniae* biofilms may have functional consequences, as C1q protects not only from pneumococcal pneumonia and sepsis (10) but also from acute otitis media and invasive disease by avoiding the dissemination of *S. pneumoniae* from the middle ear to the systemic circulation (31). Our results demonstrate that biofilm formation confer to *S. pneumoniae* an enhanced ability to circumvent the early activation of this pathway by a C1q-dependent mechanism, which is consistent with previous results reported with *Acinetobacter baumannii* (17).

The classical pathway can also be activated on *S. pneumoniae* by acute phase proteins such as CRP (22). CRP is the main acute phase reactant in humans and indeed, CRP levels markedly increase after pneumococcal infection, which confirms the importance of this molecule for *S. pneumoniae* recognition (32). Our findings show that biofilm formation in *S. pneumoniae* is associated with a reduced recognition by human CRP. This is in agreement with that previously reported for coagulase-negative staphylococcal biofilms (33) but contrasts with the claim that an enhanced production of

338 choline phosphate, which is known to bind CRP, occurs during biofilm development

339 (34). Our results suggest that the impaired activation of the classical pathway on the

340 surface of pneumococcal biofilms is not due to differences in phosphorylcholine or

341 variation in the binding to IgG. There are evidences confirming that CRP binds the

342 complement component C1q through its globular head region activating therefore the

343 classical pathway (35, 36). In this sense, our results indicate that *S. pneumoniae*

344 growing as biofilms have the ability to avoid the direct interaction of C1q with the

345 pneumococcal surface as a recent study has demonstrated that C1q can directly

346 recognize *S. pneumoniae* in the absence of any mediator (37).

347   Recruitment of regulators for complement activation is a common strategy used by

348 different microorganisms for complement evasion (38). The PspC protein of *S.*

349 *pneumoniae* binds FH reducing the activation of the alternative complement pathway

350 (27). Our results demonstrated that recruitment of FH was markedly enhanced by

351 pneumococcal biofilms compared to their planktonic counterparts in a PspC-dependent

352 manner and are in accordance with a recent report showing that pneumococci increase

353 the production of PspC when grown under biofilm forming conditions (34). Our

354 findings demonstrate that the increased presence of PspC on the surface of

355 pneumococcal biofilms have functional consequences in terms of subversion of

356 complement mediated immunity by reducing the activation of the alternative pathway

357 through a FH dependent mechanism. In contrast, a significant difference in the

358 deposition of C4BP onto biofilms as compared to planktonic pneumococcal cultures

359 was not apparent. A variety of bacteria interact with C4BP to facilitate immune evasion

360 (reviewed in reference (39). It has been reported that the binding of C4BP to *S.*

361 *pneumoniae* is PspC allele-dependent, being the R6/D39 allele a weak binder (40).

362 More recently, however, it has been reported that the pneumococcal glycolytic enzyme

363 enolase, a moonlighting surface protein (41), recruited C4BP, but not FH (39). Previous

364 proteomic analyses using procedures different to that employed in this study to grow

365 pneumococcal biofilms have show either a transient increase (42) or a marked inhibition

366 in enolase biosynthesis (43). This discrepancy has been attributed to the different strains

367 used, the different age of the biofilms examined, and/or to differences in the criteria

368 used for protein identification in both studies. Assuming that significant changes on the

369 binding of C4BP to biofilm- and planktonic-grown cells have not been found, we

370 propose that no major alterations on enolase production take place under our

371 experimental conditions. Overall, our study shows for the first time that *S. pneumoniae*

372 biofilms avoid complement immunity by targeting both the classical and alternative

373 pathway using a complex mechanism of impaired activation and increased down-

374 regulation, respectively.

375 Clearance of *S. pneumoniae* by professional phagocytes requires an efficient

376 opsonization of the bacterium by the complement system (8). Biofilm formation has

377 been suggested to be a pivotal event in the pathogenesis process of numerous infectious

378 diseases (5) and is consistent with our findings as long as the reduced complement

379 activation on *S. pneumoniae* biofilms confers a significant benefit to the virulence of the

380 microorganism. Reduced phagocytosis has been previously documented for other

381 bacterial species growing as biofilms, confirming that sessile communities of certain

382 microbial pathogens are more resistant to opsonic killing by host phagocytes than

383 planktonic cells (15, 44, 45). In the case of *S. pneumoniae,* biofilm matrices consist of a

384 mixture of extracellular polymeric substances composed of extracellular DNA, proteins

385 and polysaccharides that are synthesized in large part by the pneumococcal strains

386 producing the biofilm. The relevance of these matrices is because they are responsible

387 for the cohesion and three-dimensional architecture of biofilms (46). In terms of host-

388 pathogen interaction, the presence of an extracellular matrix during biofilm formation

389 improves the virulence of *S. pneumoniae* (47). The results of our study fully confirm

390 that opsonophagocytosis of pneumococcal biofilms was significantly impaired in

391 comparison to the planktonic cultures and demonstrate that pneumococcal biofilms have

392 developed an increased resistance to the phagocytosis process mediated by human

393 neutrophils. Taken together, our findings suggest that biofilm formation may constitute

394 an evolutionary advantage in certain phases of the pneumococcal pathogenic process,

395 such as nasopharyngeal colonization or during the early steps of microbial attachment

396 for invasion, by avoiding the host immune system.

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557

558

560

561 **FIG 1** C3b deposition on the surface of the R6 strain grown as planktonic culture (PK)

562 or as a biofilm (BF) (A). Results are expressed as a relative % FI relative to the results

563 for PK culture. (B) Example of a flow cytometry histogram for C3b deposition. A

564 control (CT PBS) incubated with PBS instead of human serum is also shown. (C) C3b

565 deposition on the surface of the encapsulated clinical isolate of serotype 19A grown as

566 planktonic culture (PK) or as a biofilm (BF) (D). Results are expressed as a relative %

567 FI relative to the results for PK culture. Error bars represent standard deviations and

568 asterisks mark results that are statistically significant compared to bacteria growing as

569 PK (two-tailed Student's *t* test, *P <0.001).

570

571 **FIG 2** C3b deposition on the surface of the R6 strain grown as planktonic cultures or as

572 biofilms. A planktonic culture of R6 strain was stained with SYTO 59 (A, red) and C3b

573 deposition on the surface of the planktonic culture of the R6 strain was visualized using

574 a FITC-conjugated polyclonal goat anti-human C3b antibody (B, green). To enhance the

575 quality of the picture, the culture was centrifuged and gently resuspended in PBS after

576 labeling and prior to examination at the fluorescence microscope (C–E) Localization by

577 CLSM of the human C3b component on the surface of biofilm-grown R6 strain. A

578 biofilm of the *S. pneumoniae* strain R6 was stained with a combination of SYTO 59 (C,

579 red) and a FITC-conjugated polyclonal goat anti-human C3b antibody (D, green). Image

580 (E) is a merger of the two channels. Scale bars = 25 μm.

581

582 **FIG 3** Deposition of C1q, CRP and IgG on the surface of the R6 strain grown as

583 planktonic culture (PK) or as biofilm (BF) and detection of PCho in PK and BF

584 cultures. (A, C and E) Deposition of C1q, CRP and IgG respectively. (B, D and F)

585 Examples of flow cytometry histograms for the binding of C1q, CRP and IgG

586 respectively. (G) Detection of PCho on the surface of the R6 strain grown as planktonic

587 culture (PK) or as biofilm (BF). (H) Example of a flow cytometry histogram for the

588 detection of PCho. Results are expressed as a relative % FI relative to the results for PK

589 culture. Controls incubated with PBS instead of human serum (CT PBS) are also shown.

590 Error bars represent standard deviations and asterisks mark results that are statistically

591 significant compared to bacteria growing as PK (two-tailed Student's *t* test, *P <0.001).

592

593 **FIG 4** Recruitment of down-regulators of the complement system by the R6 strain

594 grown as planktonic culture (PK) or as a biofilm (BF). (A) Recruitment of C4BP by the

595 R6 strain as PK (open bar) or as BF (grey bar). (B) Example of a flow cytometry

596 histogram for the deposition of C4BP on R6 strain. (C) Recruitment of FH by the R6

597 strain as PK (open bar) or as BF (grey bar). (D) Example of a flow cytometry histogram

598 for the deposition of FH on R6 strain. (E-F) Recruitment of FH by the P064 (R6 *pspC*)

599 strain as PK (open bar) or as BF (grey bar). (G) Example of a flow cytometry histogram

600 for the recruitment of FH on P064 strain. Results are expressed as a relative % FI

601 relative to the results for PK culture except in panel F, where results indicate the mean

602 fluorescence intensity. Error bars represent standard deviations and asterisks mark

603 results that are statistically significant compared to bacteria growing as PK (two-tailed

604 Student's *t* test, *P <0.001). Controls incubated with PBS instead of human serum are

605 also shown (CT PBS).

606

607 **FIG 5** Effect of PspC in complement evasion and levels of PspC in planktonic culture

608 (PK) and biofilm (BF). (A) Deposition of C3b on the surface of the R6 strain or its

609 isogenic *pspC* mutant strain growing both as biofilms**.** (B) Example of a flow cytometry

610 histogram for the deposition of C3b on R6 and P064 (R6 *pspC*) strains as BF. (C)

611 Deposition of C3b on the surface of the *pspC* strain growing as PK or BF. (D) Example

612 of a flow cytometry histogram for the deposition of C3b on P064 (R6 *pspC*). (E)

613 Detection of PspC on the bacterial surface of R6 strain growing as PK or as BF. (F)

614 Example of a flow cytometry histogram for the detection of PspC on R6 growing as PK

615 or as BF. Error bars represent standard deviations and asterisks mark results that are

616 statistically significant compared to bacteria growing as PK (two-tailed Student's *t* test,

617 *P <0.001). Controls incubated with PBS instead of human serum are also shown (CT
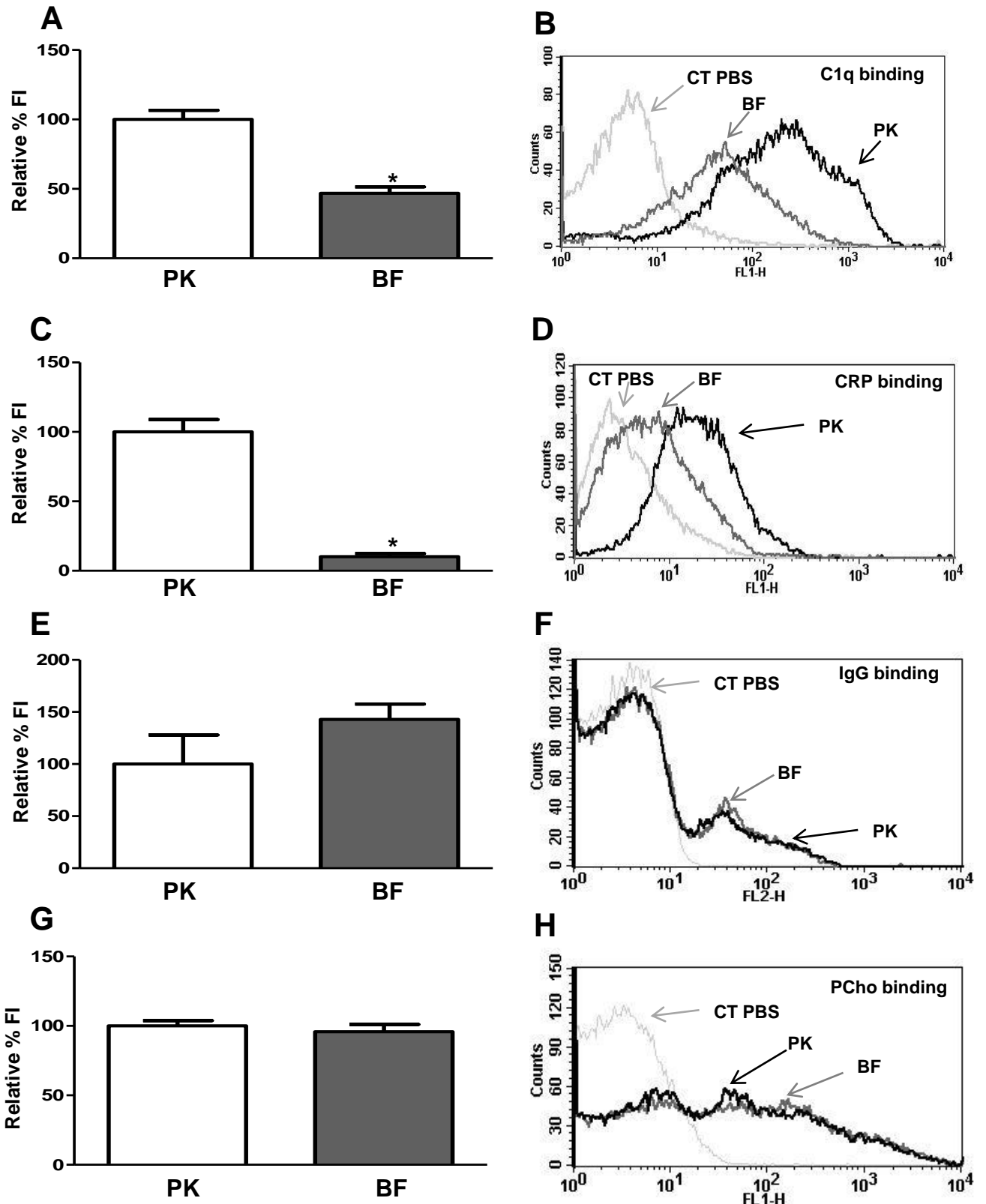
618 PBS).

619

620 **FIG 6** Opsonophagocytosis of the R6-GFP strain grown as planktonic culture (PK) or

621 as a biofilm (BF). (A) Opsonophagocytosis of the P040 strain as PK (open bar) or as BF

622 (grey bar). Negative controls of bacteria incubated with HBSS or heat inactivated serum

623 (HIS) instead of human serum are also shown. (B) Example of a flow cytometry

624 histogram for the opsonophagocytosis. Results are expressed as a relative % FI relative

625 to the results for PK culture. Error bars represent standard deviations and asterisks mark

626 results that are statistically significant compared to bacteria growing as PK (two-tailed

627 Student's *t* test, *P <0.001).

628

629

Figure 1

A

B

CT PBS

BF

PK

C3b binding

C

D

CT PBS

PK

BF

C3b binding
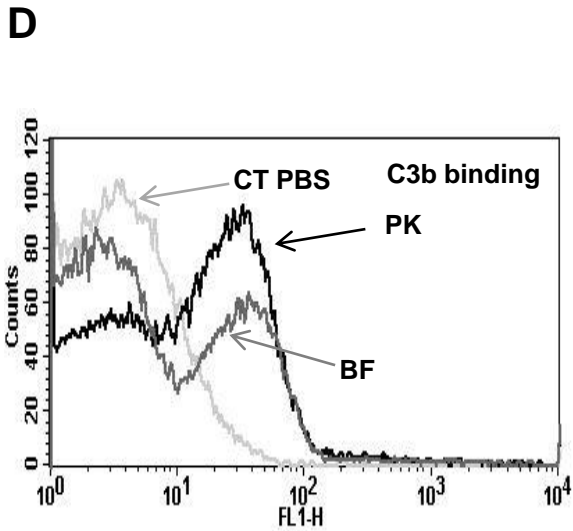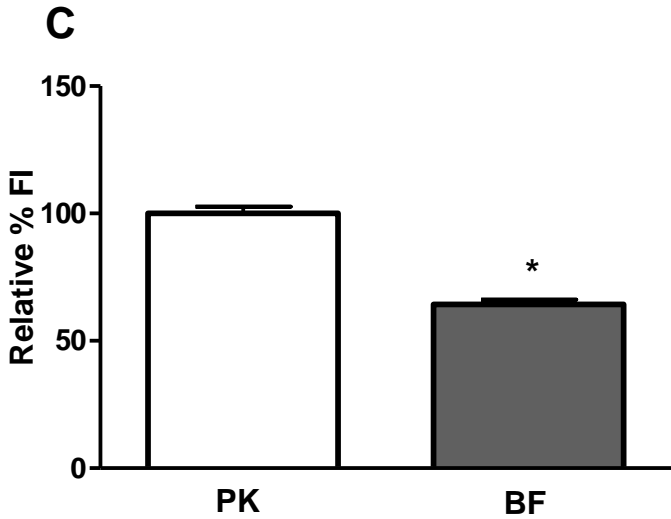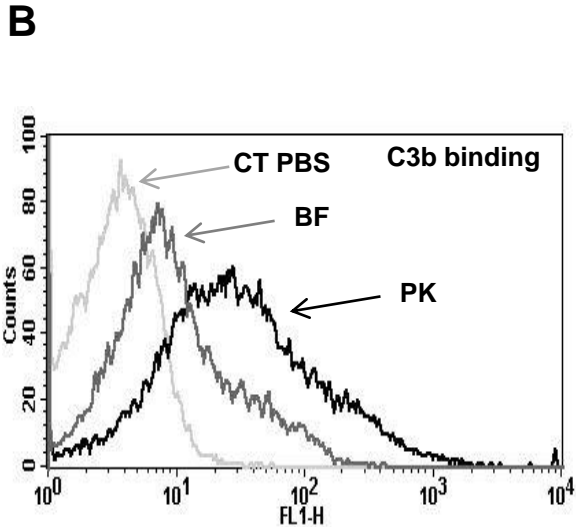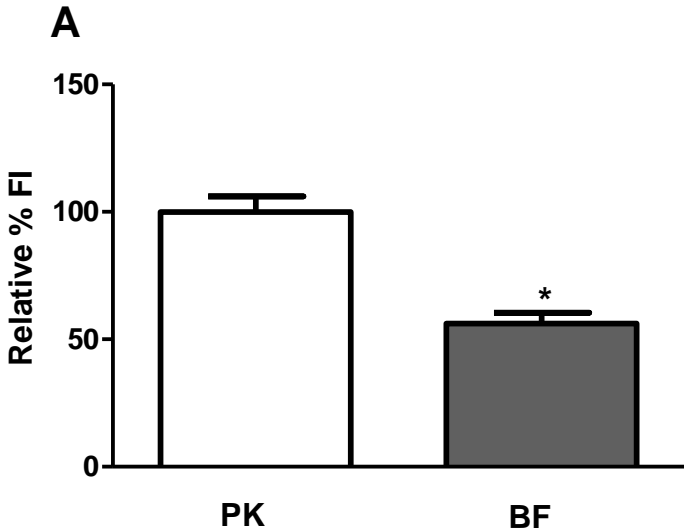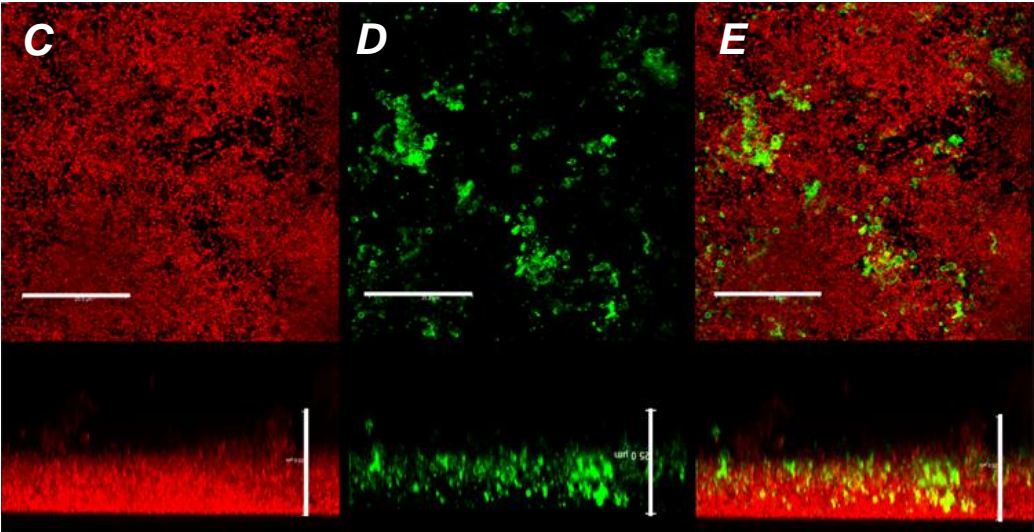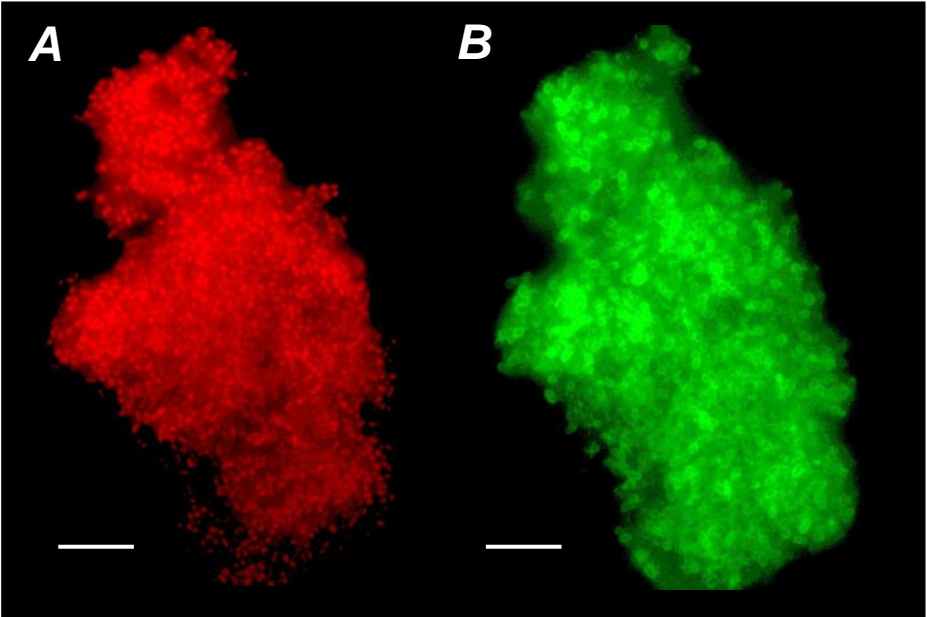
Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A

B