UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Scienze Biomediche Sperimentali

SCUOLA DI DOTTORATO DI RICERCA IN: BIOSCIENZE
INDIRIZZO: BIOLOGIA CELLULARE
CICLO XXII°

TITOLO TESI

Structural and functional characterization of second pilus type in *Streptococcus pneumoniae*

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Index

Riassunto

Streptococcus pneumoniae è uno dei più importanti patogeni che causa malattie e mortalità in tutto il mondo, quali infezioni del tratto respiratorio, otite media, setticemia, polmoniti e meningiti.

Sebbene pneumococco fosse un batterio studiato con attenzione già dal 19esimo secolo, solo recentemente si sono scoperte delle strutture dette pili che protrudono dalla superficie del patogeno. Come si riscontra in letteratura, i pili sono importanti fattori di virulenza sia nei batteri Gram-positivi che nei Gram-negativi, quest'ultimi più studiati. Al contrario, si sa ancora poco delle strutture e delle possibili funzioni dei pili nei batteri Gram-positivi.

Analizzando i genomi completi disponibili di *S. pneumonie*, abbiamo individuato una regione di 6,575 bp, che abbiamo chiamato pilus islet 2 (PI-2), contenente geni tipici di isole codificanti per pili nei Gram-positivi. In particolare, all'interno dell'isola abbiamo identificato 5 geni: due che codificano per due proteine ancorate alla superficie batterica tramite il motivo LPXTG (PitA, PitB), uno che codifica per una signal peptidasi (SipA) e due per due sortasi (rispettivamente SrtG1 e SrtG2). Tramite esperimenti di Western blot, FACS analisi ed immuno elettro microscopia (IEM) condotti su mutanti isogenici difettivi per l'espressione dei singoli componenti dell'isola, abbiamo dimostrato che PitB rappresenta lo scheletro del pilo, mentre la sortasi SrtG1 e la signal peptidasi SipA sono necessarie per l'assemblaggio e la polimerizzazione del pilo stesso. L'espressione di PitA, ipotetica proteina ancillare del pilo, risulta invece controversa. Infatti, il gene contiene uno stop codon nei nove ceppi sequenziati ad eccezione di uno in cui il gene è presente con il codone di stop seguito da un frameshift. Esperimenti di western blot eseguiti su preparati batterici trattati con mutanolisina e supernatanti concentrati di ceppi WT e relativi mutanti hanno evidenziato la presenza di una banda. Tale banda, assente nel knock-out di *pitA,* e' compatibile con l'espressione della proteina intera nel WT indicando, come già riportato in letteratura, che lo stop codon potrebbe essere letto come triptofano. Ulteriori esperimenti sono stati eseguiti per accertare l'effettiva espressione di PitA,

tramite l'utilizzo di diverse tecniche e nuovi anticorpi ottenuti contro diverse porzioni della proteina.

Inoltre, allo scopo di effettuare una caratterizzazione strutturale del pilo2 mi sono occupata della purificazione del pilo2 in forma nativa a partire da supernatanti di coltura concentrati. Esperimenti di immuno elettro microscopia, effettuati usando anticorpi α-PitB, su batteri cresciuti ON, hanno evidenziato che il pilo di tipo 2 (PI-2), a differenza del pilo di tipo 1 (PI-1), è presente in singola copia sulla superficie dei batteri e sembra essere più spesso e più corto.

Abstract

Streptococcus pneumoniae is one of the most important human pathogens and a major cause of morbidity and mortality worldwide, causing respiratory tract infections, community acquired pneumonia, and invasive diseases.

Although the pneumococcus is a well studied bacterial pathogen, first described in the late 19th century, pili on its surface were discovered only recently. Pili are elongated structures protruding from the bacterial surface and found to be important virulence factors in both Gram-positive and Gram-negative bacteria. In contrast to Gram-negative pili, little was known about the structure of Gram-positive pili. In the recent years the structure and function of the first pilus type (pilus-1) has been elucidated and a second pilus type (pilus-2) in *S.pneumoniae* has been characterized. Here we describe the genomic organization of a second PI, PI-2, identified in the partial genome sequence of a serotype 1 *S. pneumoniae* strain (INV104). Furthermore we provide experimental evidence that PitB is the backbone protein forming the pilus-2 shaft. We also confirm that both the sortase SrtG1 and the signal peptidase-related protein SipA, are necessary for assembly and polymerization of the pilus. In order to study the pneumococcal pilus in detail, a purification procedure was set up to obtain pure native pili preparations. Pneumococcal pili were isolated from strain PN110, bacteria that in low-dose EM showed individual pili and bundles of individual pili on the bacterial cell surface.

CHAPTER1

General introduction

1.1 *Streptococcus pneumoniae*

The Gram-positive bacterium *Streptococcus pneumoniae*, also known as pneumococcus, is one of the most important human pathogens causing respiratory tract infections such as sinusitis, otitis media, and community acquired pneumonia, but also invasive diseases such as septicemia and meningitis. Together with HIV, malaria, and tuberculosis the pneumococcus represents one of the four major infectious disease killers. A major virulence factor of *Streptococcus pneumoniae* is the polysaccharide capsule, by which pneumococci are grouped into at least ninety different serotypes. Other genetic factors, such as CbpA (choline-binding protein A) and pneumolysin, have been described to be of importance for virulence. Infection by *Streptococcus pneumoniae* leads to invasive disease triggered by initial colonization of the nasopharynx, but the mechanisms of adhesion are still not well understood.

Moreover, the World Health Organization estimates that over 90% of these deaths occur in developing countries. Approximately, 1 million children under 5 years of age die of pneumococcal disease annually. In countries where the incidence of *Neisseria meningitidis* and *Haemophilus influenzae* infections has drastically decreased through the introduction of vaccines against meningococci group C and *H. influenzae* type B, *S. pneumoniae* has become the major cause of meningitis and septicemia in children. In addition, the morbidity by *S. pneumoniae* through respiratory tract infections such as otitis media and sinusitis is enormous. Thirty to 50% of all patients with otitis media and a substantial percentage of cases of sinusitis and pneumonia are caused by pneumococci. Risk groups for serious pneumococcal disease include children under the age of 2 years, elderly and patients with immunodeficiencies. Despite this high burden of disease, the pathogenic mechanisms exploited by *S. pneumoniae* are not yet clear. A critical step is colonization of the nasopharynx and the initial interaction of pneumococci with host cells.

Nasopharyngeal colonization by *S. pneumoniae* is common: probably all humans are colonized with this organism at least once early in life. Especially in circumstances of crowding, as in day-care centers, nursing homes, hospitals and jails, the risk of colonization with pneumococci is high. Colonization is not usually followed by

disease, since this is prevented by the innate and adaptive immune system. However, disturbance of homeostasis between host and pathogen, for example through viral infections, malnutrition or local damage of the mucosa, is associated with the development of (invasive) diseases.

Since the discovery of the antibacterial properties of penicillin by Fleming in 1929, many antibiotics have been used for treatment of pneumococcal infections. Recently, antibiotic resistance has become a worldwide problem, which limits the choice of antimicrobial agents. Therefore, prevention of pneumococcal disease has become of great interest.

The pneumococcal outer surface consists of a cell wall covered by a polysaccharide capsule. As said before, capsule polysaccharides are highly heterogeneous and, thus far, almost 100 different capsular serotypes have been described. The polysaccharide capsule is the most important virulence factor of the pneumococcus as it protects the bacteria from phagocytosis. Capsular polysaccharides are highly immunogenic and antibodies against these polysaccharides protect against infection with the homologous serotype. The antigenicity of the capsule is type-specific, but cross-reactions occur because of shared polysaccharides. The next layer, the cell wall, consists of polysaccharides, teichoic acid and several cell walls associated surface proteins. The cell wall is responsible for the intense inflammatory reaction that accompanies pneumococcal infection since it stimulates the influx of inflammatory cells. In addition, it activates the alternative complement cascade and induces cytokine production. The cell-surface associated proteins are believed to specifically contribute to virulence as well. The most immunogenic part of the cell wall is the phosphocholine part of the teichoic acid, which is also playing a major role in the inflammatory process. The cell wall is shielded from the host response by the capsule which is completely covering it. Pneumococcal clearance from the lung mainly results from phagocytosis and intracellular killing of the bacteria by neutrophils and alveolar macrophages. This process can only occur in the presence of type-specific immunoglobulins (IgG1 and IgG2, IgM and IgA) and active complement. This antibody-initiated complement-dependent opsonization, which activates the classical complement pathway, is believed to be the major immune mechanism protecting the

host against infections with pneumococci. Pneumococci may escape this mechanism in the absence of serotype-specific antibodies, and consequently, may enter the host through the interstitial tissue of the lung resulting in lymphatic spread and subsequent blood stream invasion causing bacteremia. The mechanism of clearance from the blood appears to depend on the interaction of type-specific antibodies (IgG) complement and phagocytic cells in the liver and spleen. The absence of the spleen or cirrhosis of the liver predisposes for severe pneumococcal infection. Congenital deficiencies in immunoglobulin or complement are also associated with predisposition to pneumococcal infection. Non-capsular antibodies, for example immunoglobulins directed against cell wall components, may play a role in the host response to pneumococcal infection as well. Although most cell wall components are protected from opsonization and phagocytosis by the capsule, certain proteins may penetrate the capsule and may therefore be recognized by the immune system.

So far, several animal studies have shown a protective effect of immunoglobulins directed against selected cell surface-associated proteins including pneumococcal surface protein A (PspA), pneumococcal surface adhesin A (PsaA), and pneumolysin. This T cell dependent immune response (TD), which is present from birth, induces mainly IgG1 subclass immunoglobulins. Type-specific immunoglobulins may be more effective if from the IgG2 subclass. The adult antibody response against capsule polysaccharides mainly generates these IgG2 subclass antibodies. However, the antibody response to pneumococcal polysaccharides is relatively immature in young children and generates predominantly IgG1 antibodies, which may result in an enhanced susceptibility to pneumococcal infections. Thus, with the type of vaccination, the type of immune response may differ, and so may the level of protection. One has to take this into account when evaluating different future preventive strategies.

1.2 Pili in Gram-negative and Gram-positive bacteria

Several types of surface appendages have been identified in both Gram-negative and Gram-positive bacteria. They perform different bacterial function during bacterial life cycle, such as biofilm formation, cell aggregation, host cell invasion, DNA transfer and motility. Accordingly, their structure has to assure both resistance and flexibility necessary to play their roles in the different physiological environments. The role of pili as adhesive organelles is particularly important for pathogenic bacteria because they have to attach to specific host cells during colonization. For a number of bacterial pathogens, pili-like appendages are reported to be virulence factors and several works describe them as targets for vaccine development. While Gram-negative pili have been studied in detail over the last decades, the majority of Gram-positive pili have only recently been discovered. In contrast to Gram-negative pili, which are formed by non-covalently linked subunits, Gram-positive pili studied so far are extended polymers formed by a transpeptidase reaction involving covalent cross-linked subunit proteins containing specific amino acid motifs, which are assembled by specific sortases. Sortases are also responsible for the covalent attachment of the pilus to the peptidoglycan cell wall.

In Gram-negative bacteria, pili are typically formed by non-covalent interactions between pilin subunits. By contrast, the recently discovered pili in Gram-positive pathogens are formed by covalent polymerization of adhesive pilin subunits. Evidence from studies of pili in the three principal streptococcal pathogens of humans indicates that the genes that encode the pilin subunits and the enzymes that are required for the assembly of these subunits into pili have been acquired *en bloc* by the horizontal transfer of a pathogenicity island. Non-flagellar appendages were first observed in bacteria in the early 1950s, while the outer-membrane surface of Gram-negative pathogens was being scanned with the electron microscope. During the following decade, these filamentous structures were recognized and characterized for several Gram-negative species by two research groups: one led by James Duguid, who referred to these structures as fimbriae2 (derived from the Latin word for fringe); and the other led by Charles Brinton, who preferred to call these structures pili (derived

from the Latin word for hair or fur). To this day, the two terms, fimbriae and pili, are still in use, even though they are synonymous.

Over the past five decades, several distinct pilus types have been identified, most of which were described and characterized in Gram-negative bacteria. The best characterized of these cell-surface organelles are type I pili (expressed by enteropathogenic *Escherichia coli*), type IV pili (expressed by *E. coli*, and *Pseudomonas* and *Neisseria* species) and curli pili, (expressed by some strains of *E. coli*). Under the electron microscope, type I pili appear as peritrichous, rigid, rod-like structures of 1–2 μm in length, and they have a visibly flexible tip that is known to be involved in bacterial interaction with receptors on the host-cell surface. Type IV pili are a similar length but differ from type I pili in that they appear to be more flexible and often form bundles at polar locations. Curli pili are, as their name suggests, coiled structures. All three pilus types are formed by the non-covalent association of pilin subunits into regular polymeric structures. Pilus assembly in Gram-negative bacteria has been well studied and involves the Sec-dependent secretion of the main (backbone) and tip pilin subunits into the periplasmic space, where chaperones prevent them from folding correctly until they reach the outer membrane, which is the site of assembly. The best-studied pilus types are generated by one of the following mechanisms: the chaperone/usher pathway (type I pili), a type II secretion system (type IV pili) or nucleation-dependent polymerization (curli pili). Different types of pilus can have different roles. For example, both type I and type IV pili are involved in adherence to host cells and induction of signaling in these cells. By contrast, only type IV pili allow the transfer of genetic material, and they are also known to retract, thereby producing a moving force on the bacterium that is known as twitching motility. A common feature of Gram-negative pili, however, is their role in adhesion to eukaryotic cells. It has been proposed that bacteria use these structures to form an initial association with host cells, which can then be followed by a more ‘intimate’ attachment that brings the bacterium into proximity to the host-cell surface. Pili are known to adhere to components of the extracellular matrix , as well as to carbohydrate moieties that are present in glycoprotein or glycolipid receptors.

Receptor specificity might be important in determining the specificity and tropism of bacteria for particular host cells.

Pilus-like structures on the surface of Gram-positive bacteria were first detected in *Corynebacterium renale*, by electron microscopy. More recently, surface appendages were reported to be present in *Actinomycesnaeslundii* and were subsequently found in other species, including *Corynebacterium diphtheriae*, *Streptococcus parasanguis*, *Streptococcus salivarius* and *Streptococcussanguis* .

Finally, in the past year, pili were also characterized in all three of the principal streptococcal pathogens that cause invasive disease in humans — group A *Streptococcus* (GAS; that is, *Streptococcus pyogenes*), group B *Streptococcus* (GBS; that is, *Streptococcus agalactiae*) and *Streptococcus pneumoniae*— in which they have been shown to have key roles in the adhesion and invasion process and in pathogenesis.

1.3 First pilus type in *Streptococcus pneumoniae*

Recently, type I pili have been detected on the cell surface of *Streptococcus pneumoniae* and identified as important virulence factors involved in adhesion and biofilm formation. Pneumococcal pili, which are present in some but not all clinical isolates, are encoded by the *rlrA* islet, which includes the genes for the three pilus subunits (RrgA, RrgB, and RrgC). These elongated structures up to 1 µm in length and 6nm in diameter have been reported to form coiled-coil superstructure formed by several copies of the backbone subunit RrgB and two ancillary proteins RrgA and RrgC (see Hilleringman et al. 2008. PLoS Pathog).

In particular, triple immunoelectron microscopy of the elongated structure showed that purified pili contained RrgB as the major compound, followed by clustered RrgA and individual RrgC molecules on the pilus surface. The arrangement of gold particles displayed a uniform distribution of anti-RrgB antibodies along the whole pilus, forming a backbone structure. Antibodies against RrgA were found along the filament as particulate aggregates of 2–3 units, often colocalised with single RrgC subunits. Structural analysis using cryo electron microscopy and data obtained from freeze drying/metal shadowing technique showed that pili are oligomeric appendages formed by at least two protofilaments arranged in a coiled-coil, compact superstructure of various diameters. Using extracellular matrix proteins in an enzyme-linked immunosorbent assay, ancillary RrgA was identified as the major adhesin of the pilus. Combining the structural and functional data, a model emerges where the pilus RrgB backbone serves as a carrier for surface located adhesive clusters of RrgA that facilitate the interaction with the host. Furthermore, in a mouse model of intraperitoneal infection Gianfaldoni et al. reported protective immune responses after active and passive immunization with recombinant pilus subunits of Streptococcus pneumoniae Type 4 strain TIGR4 (T4). Analysis of native pneumococcal pili revealed structural basics of a Gram-positive pilus that could also serve as a basis for effective vaccine design.

1.4 Second pilus type in *Streptococcus pneumoniae*

Very recently the analysis of genomes of *S. pneumoniae* has led to the identification of a new genomic element containing genes typical of Gram-positive pilus islets (PI) that code for a second functional pilus able to polymerize and to adhere to host tissues.

In this study I describe and characterize the genetic organization of a second PI, named PI-2, identified in the partial genome sequence of a serotype 1 *S. pneumoniae* strain (INV104). Detailed structural studies have been also performed and the obtained data suggest that this pneumococcal PI favors bacterial adhesion to host tissues. The presence of different pilus types reinforces the notion that these structures, being evolutionarily rewarded, may confer a critical selective advantage also for the pneumococcus.

Presence of PI-2 correlates with the genotype as defined by multilocus sequence typing and clonal complex (CC). The PI-2-positive CCs are associated with serotypes 1, 2, 7F, 19A, and 19F, considered emerging serotypes in both industrialized and developing countries. Interestingly, strains belonging to CC271 (where sequence type 271 is the predicted founder of the CC) contain both PI-1 and PI-2, as revealed by genome analyses. In these strains both pili are surface exposed and independently assembled. Distribution of PI-2 in a global collection of clinical isolates was assessed to be 16%, with very high sequence conservation. By Immunogold labeling (IEM) the second pilus type (pilus-2) was characterized. It appears different in morphology with respect to the pilus-1. Pilus-2 is present in single copy per bacterium and it is thicker than pilus-1. We also found in pilus-2 a structure similar of that one of conjugative pili in typical Gram-negative bacteria (Trudy H. Grossman, J. Bacteriology, 1990). This reason lead us think that pilus-2, on the contrary of pilus-1 that has an important role in adhesion, could be or could be involved in conjugation.

CHAPTER 2

Structural and functional characterization of second pilus type in *Streptococcus pneumoniae*

Results

Some of the following results have been published in the article:

F. Bagnoli, M. Moschioni, C. Donati, V. Dimitrovska, I. Ferlenghi, C. Facciotti, A. Muzzi, F. Giusti, C. Emolo, A. Sinisi, M. Hilleringmann, W. Pansegrau,S. Censini, R. Rappuoli, A. Covacci, V. Masignani and M. A. Barocchi. A Second Pilus Type in *Streptococcus pneumoniae* Is Prevalent in Emerging Serotypes and Mediates Adhesion to Host Cells. JOURNAL OF BACTERIOLOGY, Aug. 2008, p. 5480–5492

2.1 Genomic organization of PI-2 in *S. pneumoniae*

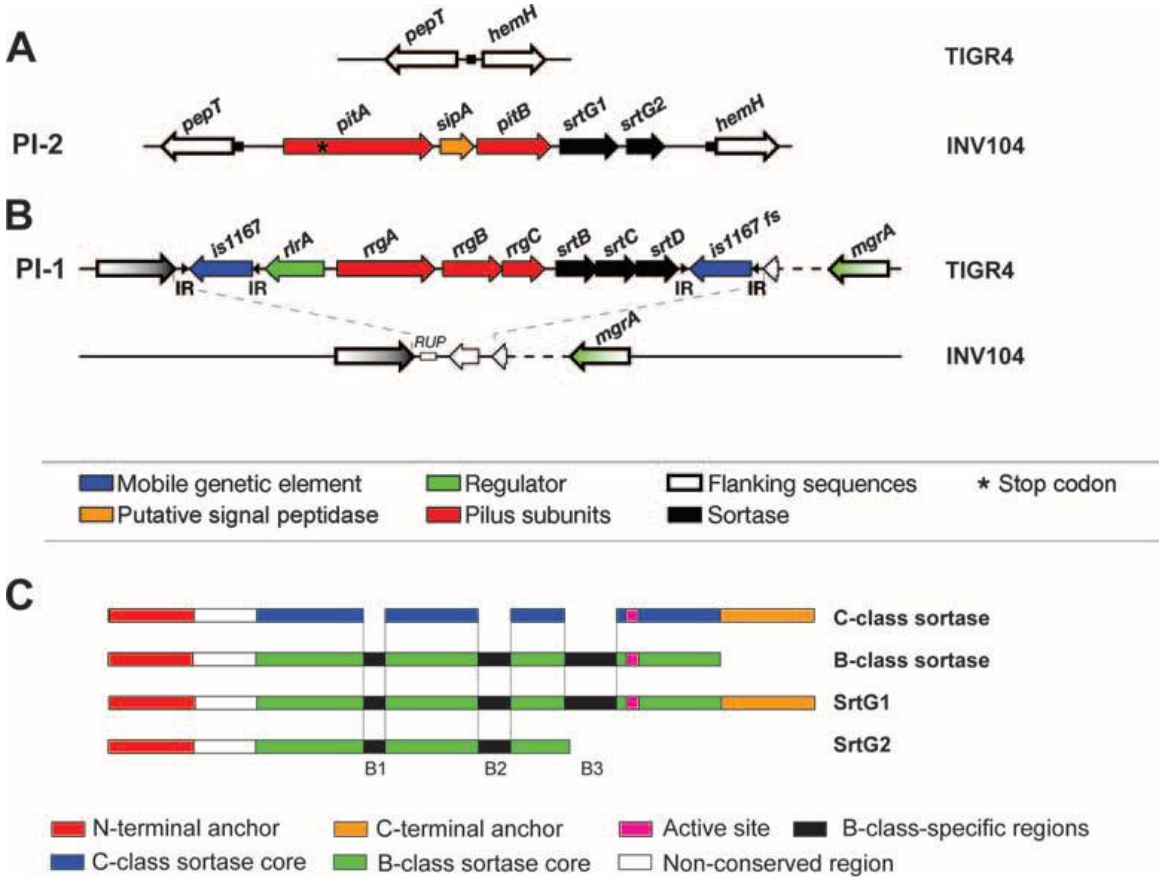


FIG. 1. The genomic organization of pilus-encoding islets in *S. pneumoniae*. Schematic representation of PI-2 (A) and PI-1 (B) genomic regions in TIGR4 and INV104 strains. TIGR4 is positive for PI-1 and negative for PI-2, whereas INV104 is positive for PI-2 and negative for PI-1 presence. Genes coding for proteins with different roles are represented with different colors, as shown. (C) Schematic representations of the architectures of class B and C sortases and comparison with those of SrtG1 and SrtG2. Functional elements are shown in different colors, as indicated.

In the search for additional pilus structures, we analyzed all the publicly available *S. pneumoniae* genome sequences and found a region whose organization resembled the pilus-encoding islets in *S*. *pyogenes* (group A streptococcus) in the serotype 1 INV104 strain (www.sanger.ac.uk). This newly identified genetic region, herein named PI-2, is composed of five genes: two genes encoding products displaying similarity to sortases (*srtG1* and *srtG2*), a gene encoding a signal peptidase-related product (*sipA*), and two genes coding for putative LPXTG-type surface-anchored proteins (*pitB* and *pitA*, the latter containing a stop codon) (Fig. 1A).

PI-2 is 6,575 bp in length and has a G_C content of 38.5%, similar to the overall G_C content of *S. pneumoniae*. In the strains where the islet is absent, the flanking genes *pepT* and *hemH* are separated by a noncoding region of approximately 140 bp that contains a putative 7-bp insertion site (TCCTTTT), which is found duplicated at the boundaries of PI-2 (Fig. 1A). INV104 does not contain any remnants of PI-1 at the locus that corresponds to the site where PI-1 is inserted in TIGR4 (Fig. 1B). Homology searches revealed that PitB and PitA share 27% and 25% sequence conservation, respectively, with the backbone and ancillary proteins of a pilus produced by *S*. *pyogenes*. These data suggest that PitB is the most probable backbone of the pilus and PitA, a putative ancillary protein. Detailed investigation of PitB and PitA amino acid sequences highlighted the presence of motifs characteristic of gram-positive pilus subunits: a signal peptide and a non-canonical CWSS containing the sequences VTPTG (PitB) or VPETG (PitA). In addition, PitB is predicted to have both a pilin motif, FKENNK SNA**PK**V, found at residue 200 and an E-box motif, **Y**TVT**ET**G VA**GY**, at the C-terminal region (highly conserved residues are indicated in boldface).

2.2 Distribution of PI-2 in a global collection of clinical isolates and its sequences conservation

In order to determine the PI-2 prevalence, a global collection of 305 clinical *S. pneumoniae* isolates was analyzed (Table 1).

Strain source	Country	No. of isolates	Diagnosis (no. of isolates)[a]	Reference or source
PMEN	Various	26	ID	24
CDC	United States	50	ID	6
Oswaldo Cruz Foundation	Brazil	55	ID (27), C (28)	
ISS	Italy	40	ID (37), C (3)	
Swiss Tropical Institute	Ghana	15	ID	17
Dhaka Shishu Hospital	Bangladesh	36	ID	
Kenyan Medical Research Center	Kenya	5	ID	
Karolinska Institute	Sweden	8	ID	
Norwegian Institute of Public Health	Norway	43	C	40a
University of Alabama	Various	13		
Novartis	Various	11	ID	
Sanger Institute	Various	3	ID	www.sanger.ac.uk

[a] ID, invasive disease; C, carriage.

TABLE 1. Composition of the strain collection used to determine PI-2 distribution.

The collection represents 43 serotypes and 148 diverse STs, which can be grouped into 70 CCs (by eBURST analyses) and which include isolates from invasive disease as well as nasopharyngeal carriage. The prevalence of PI-1 in this collection is 31.5% (96 positive isolates), confirming previously published data. PI-2 presence was determined by PCR analyses as described in the Materials and Methods section.

Overall, 50 isolates contained PI-2 (16.4%), which was found consistently inserted in the same genomic region. As previously demonstrated for PI-1, presence of PI-2 correlates with the genotype defined by CC. Furthermore, the prevalence of the PI-2 is the same in both invasive disease and carriage isolates. When the collection was stratified by serotype, the serotypes tested were not homogeneous for PI-2 presence (data not shown), confirming that there is no correlation between the PI-2 and serotype. Among the isolates tested, PI-2 was present in six different CCs (74, 191, 251, 271, 304, and 306) out of 70 tested (8.6%), corresponding to serotypes 2, 7F, 19A, 19F, and 1 (Table 2). Interestingly, only CC271 from this strain collection contains both PI-2 and PI-1.

Nine isolates representing the six positive CCs were analyzed for sequence variability of PI-2: INV104, SPPD, PN110, 31620, 32_14, 5167-99, SP231, PGX1416, and 19F Taiwan-14.

CC	Serotype	Disease outcome[c]	PI-2	PI-1	Strain name(s) (serotype, ST)
74	2	ID	Yes	No	31620 (2, 74)
191	7F	C, ID	Yes	No	32_14 (7F, 191)
251	19F		Yes	No	5167-99 (19F, 251)
271[b]	19A, 19F	C, ID	Yes	Yes	19F Taiwan-14 (19F, 236), pgx1416 (19F, 271), sp231 (19F, 271)
304	1	ID	Yes	No	PN110 (1, 304)
306	1	ID	Yes	No	INV104 (1, 227), SPPD (1, 306)

[a] CCs positive for PI-2, the serotype to which they are associated, and their provenance are shown (n = 305 isolates).
[b] CC271 was associated with both pilus-encoding islets.
[c] ID, invasive disease; C, carriage.

TABLE 2. Distribution of PI-2 in the *S. pneumoniae* strain collection.

Multiple sequence alignment of the nine PI-2s by Clustal W revealed an overall conservation of _99% along the entire islet. Further analysis of the *pitA* sequences revealed the presence of the previously mentioned stop codon (UGA) in all the isolates, as well as an additional frameshift following the stop codon in strain 5167- 99 (Table 3).

>INV104

MNVQYDFKKIQYFTSSLVIFLAILFLCAPINSLRADSTTEPQTTLHKTITPISGQEDQYELSLDITSKLGTETQTETQSEPLDVVLVADF
SGSMEERDVWSYSSRRYISRIEALKHTLKGVNGRQGLIDTILSNSQNRLSIVGFAGKIDNQYNGRYYNEYYLSYQYGTWPN*****AGW
YSNISSYDDAKTLVSWSTDSNSSKNIVSSLTIADSSHSYG**MDAGI**GTGTNINAGLTEAQRLLQSARAGAKKVVILLSDGEANMYYE
SNSGRTIYNYYSNPNVGRMIDTPYWFTSGLERGMLNISSLIAPKIDGFYSIKFRYIGSNDSITSLKGYISGYNSGIPNEIFSANNENDL
QQKFKEITDKILPLGVHHVTISDVLSKYVQLLPGDASHLRVVKIKDGNEQELNDNQVTIETKKNEQGLVEVTAKFNPSYTLEDDAKY
VLKFTVTSSQEAFDAIAGDKTLTSDDAEEADATKLYSNKGAKVAYSYGIGTSRTKIKDYSEKPTFKPSDPLTVPVEIEWKGVDGKS
NPSANRPPSVELNLNQKKDGSIKDSYRKVTSPVQTNSFTENTSFAKVAKGYDYELKAPDAPGYTVEVQKTGTKEKPSFKVIYRQL
PSLTVKKILEGEQSPNKSFTINVTLSDKDGKPINGKFGNTTVTNGKAQISLKNSQETALSYLPRDTHYKVEEVENSRTGYHVTYEK
QEGTLSEDVQTIVTNHRLPTLSVTKKVTGAFANLLQSFKITINVKDAQNKPLNGSYSAIVNNQKTTLQFTNGKATVDLKKDKTIKILD
LPLNARYSIEEEASSSRGYQVSYDKKEGTLDANKSATVTNNKNS**VPETG**IDFLSSTLVLGVVLPLGGIFFIILLGHLVVNRRK*

>5167

*TFNMILRRFNILPVV*LSFSLFFFCVHQLILYVQIQ*LNLRQLCTKRLLRYQGKKTSMSCHWISHLNWERRPSQNPWM*SWLPIFQG
VWKSEMCGLTLVDDTLVGLKH*NIH*KV*MVVRGSLIQFFLIPKTVCL*LVLPERLIISIMTVIIMNII*VINMELGQIELVGIQISLHIDDAK
TLVSWSTDSNSSKNIVSSLTIADSSRSYG**MDAGI**GTGTNINAGLTEAQRLLQSARAGAKKVVILLSDGEANMYYESNSGRTIYNYY
SNPNVGRMIDTPYWFTSGLERGMLNISSLIAPKIDGFYSIKFRYIGSNDSITSLKGYISGYNSGIPNEIFSANNENDLQQKFKEITDKIL
PLGVHHVTISDVLSKYVQLLPGDASHLRVVKIKDGNEQELNDNQVTIETKKNEQGLVEVTAKFNPSYTLEDDAKYVLKFTVTSSQE
AFDAIAGDKTLTSDDAEEADATKLYSNKGAKVAYSYGIGTSRTKIKDYSEKPTFKPSDPLTVPVEIEWKGVDGKSNPSANRPPSVE
LNLNQKKDGSIKDSYRKVTSPVQTNSFTENTSFAKVAKGYDYELKAPDAPGYTVEVQKTGTKEKPSFKVIYRQLPSLTVKKILEGE
QSPNKSFTINVTLSDKDGKPINGKFGNTTVTNGKAQISLKNSQETALSYLPRDTHYKVEEVENSRTGYHVTYEKQEGTLSEDVQTI
VTNHRLPTLSVTKKVTGAFANLLQSFKITINVKDAQNKPLNGSYSAIVNNQKTTLQFTNGKATVDLKKDKTIKILDLPLNARYSIEEEA
SSSRGYQVSYDKKEGTLDANKSATVTNNKNS**VPETG**IDFLSSTLVLGVVLPLGGIFFIILLGHLVVNRRK*

TABLE 3. PitA protein sequence analysis (Orf1290). All the 9 sequenced strains have the stop codon in pitA. Only 5167 strain has an additional frameshift following the stop codon.

Moreover, the putative sortase *srtG2* (99.2% conserved) is complete only in the isolate 5167-99, whereas, in the other isolates tested, either the C-terminal part of the gene product containing the active cysteine residue is missing, or a dinucleotide insertion in the 5_ region of *srtG2* results in a nonfunctional pseudogene.

2.3 The backbone protein PitB is surface exposed and assembled into a pilus in a SrtG1- and SipA-dependent manner

To determine if the putative cell wall-anchored PitB was expressed and assembled into a pilus structure, mouse serum was raised against a His-tagged PitB recombinant protein and used to label bacteria grown in liquid culture at mid log phase. The PI-2-

positive isolates were specifically recognized by anti-PitB antibody upon FACS analyses, whereas the isolates lacking PI-2 showed a background level of staining (Fig. 2). As expected on the basis of the high level of sequence conservation, the antibody was able to efficiently detect the protein expressed by all the isolates.

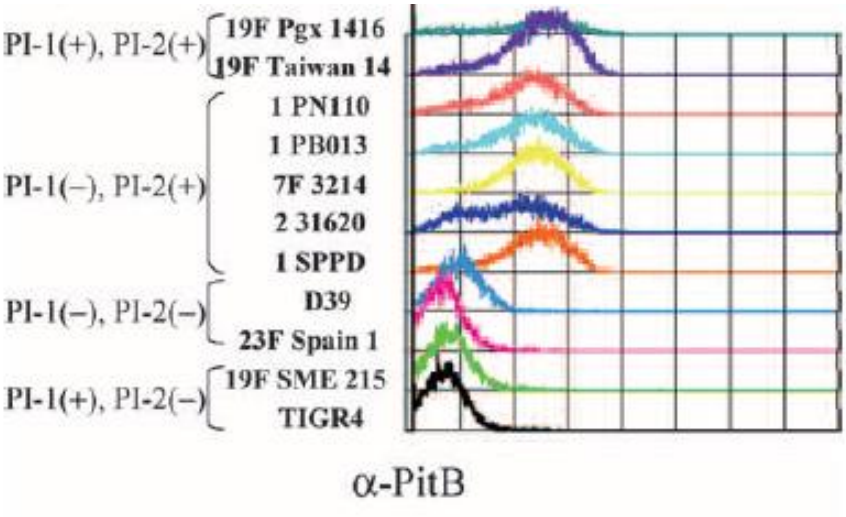


FIG. 2. Detection of a functional surface-exposed pilus structure encoded by PI-2. FACS analysis performed on *S. pneumoniae* clinical isolates (OD600 of 0.2) containing PI-1, PI-2, or both (presence or absence indicated by a plus or minus sign, respectively) labeled with mouse polyclonal PitB antiserum (secondary antibody was fluorescein isothiocyanate labeled).

In addition, immunoblotting with anti-PitB antibody on mutanolysin cell wall extracts was performed on several strains (both PI-2 positive and negative). As shown in Fig. 3, a typical HMW ladder was detected only in PI-2-positive isolates by immunoblot analysis with anti-PitB antibody. Detection of an HMW ladder is indicative for the presence of extended pili, resulting from the fact that in pili of gram-positive bacteria there is always a statistical distribution of covalently assembled pilus filaments of

various lengths, with the shorter ones entering the gel while the longer ones remain close to the starting point.

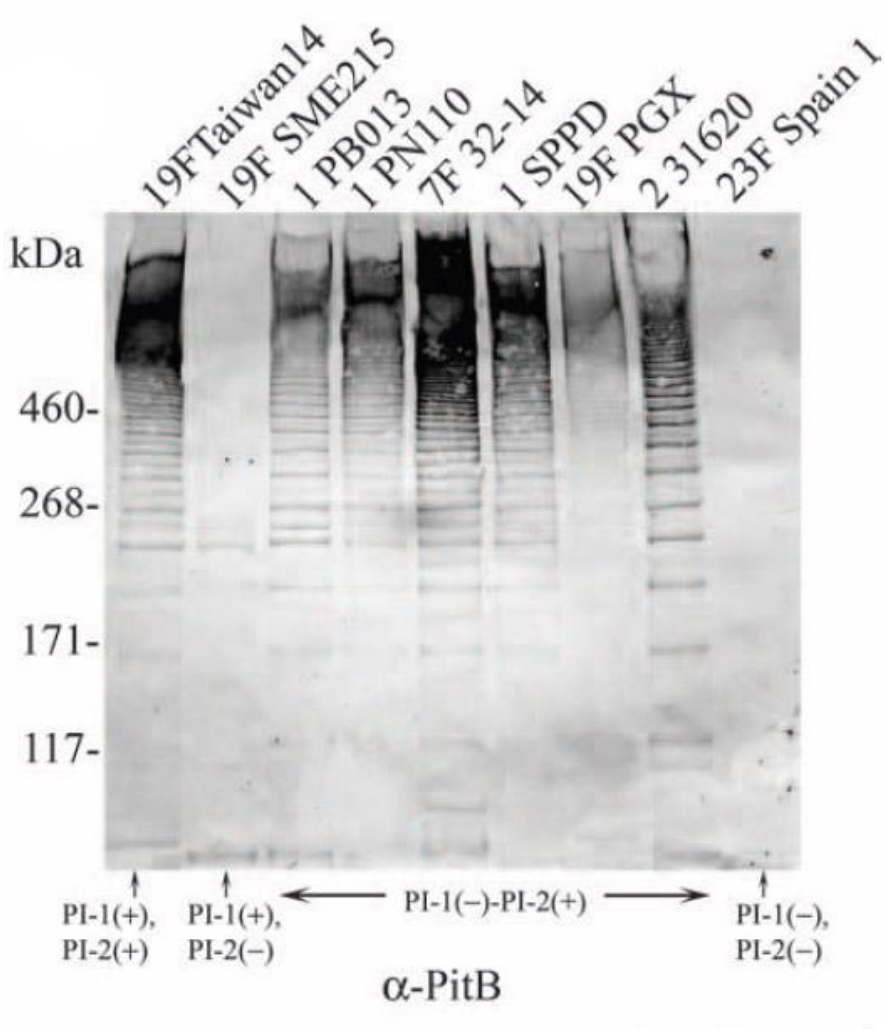
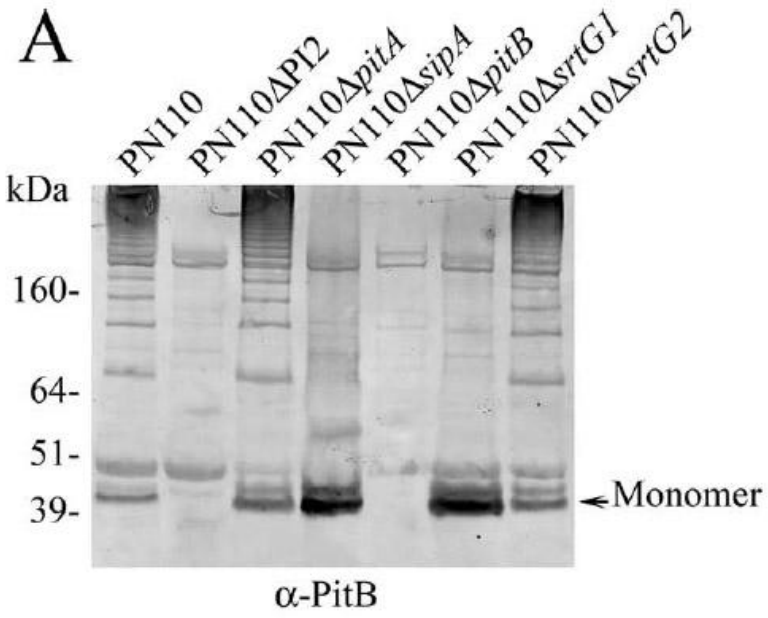


FIG. 3. Immunoblot analysis of different *S. pneumoniae* mutanolysin extracts reacted with mouse polyclonal anti-PitB antiserum.

In order to demonstrate the requirement of PI-2 and the islet-specific genes in the assembly of the pilus, a panel of mutants was constructed by insertional mutagenesis

in the strain PN110 (Table 4 in Material and Methods). Specifically, isogenic mutants containing deletions of the entire PI-2 or the individual open reading frames contained in the islet were created. PN110wt and all the isogenic mutants were subsequently analyzed both by western blot and immunogold electron microscopy.

Figure 4 shows the typical HMW ladder detected by anti PitB antibodies in Western blot experiments on mutanolysin extracts (panel A) and immunogold electron microscopy pictures of bacteria stained with anti-PitB antibody (panel B). According to the expectations, the pilus was not detected on the surface of mutant strains lacking either PI-2 (PN110_PI2) or *pitB,* confirming that the presence of the islet is necessary for pilus assembly and that *pitB* encodes for the pilus shaft (Fig. 4A and E). Furthermore, the lack of the putative sortase SrtG1 impaired the polymerization of PitB without affecting its expression (Fig. 4A and F). Indeed, in the PN110_*srtG1* mutant, the pilus backbone protein was found in mutanolysin cell extracts as a monomer, suggesting that SrtG1 acts as the sortase involved in the pilus polymerization. We observed the same results with the isogenic mutant of *sipA*, confirming recent data obtained in *S*. *pyogenes*, where the essentiality of the signal peptidase-like protein for pilus assembly has been demonstrated (Fig. 4A and D) and an alternative function as a chaperone has been proposed. In contrast, the expression or polymerization of PitB was not affected in isogenic mutants of the putative ancillary pilus subunit PitA and the sortase-like SrtG2 (Fig. 4A, C, and G). These observations, taken together, demonstrate that PitA and SrtG2 are dispensable for pilus polymerization and that; probably *srtG2* gives rise to a non-functional protein product.


A
PN110
PN110ΔPI2
PN110Δ*pitA*
PN110Δ*sipA*
PN110Δ*pitB*
PN110Δ*srtG1*
PN110Δ*srtG2*
kDa
160-
64-
51-
39-
Monomer
α-PitB

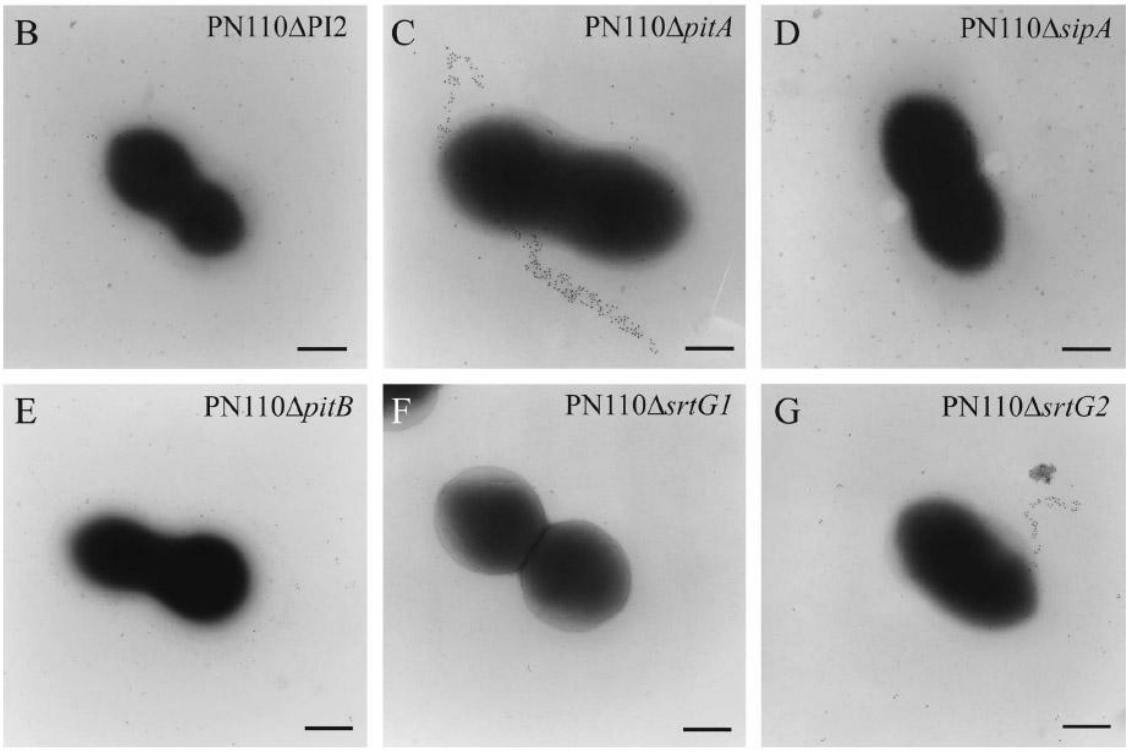


FIG. 4. Effect of PI-2 gene deletions on PI-2 polymerization in *S. pneumoniae* Serotype 1. (A) Western blot performed with anti-PitB antibodies on mutanolysin extracts of PN110 knockout isogenic mutants. The PitB monomer is indicated. (B to G) Immunogold Electron Microscopy labeling experiments with anti-PitB antibodies of whole-cell PN110 deletion mutants. Mutants PN110_PI2 (B), PN110_*sipA* (D), PN110_*pitB* (E), and PN110_*srtG1* (F) show lack of pilus formation. Panels C and G represent PN110_*pitA* and PN110_*srtG2*, respectively. Bacteria were charged on Formvar Carbon Coated Copper Grids. Scale bar 100nm.

Western blotting analysis of PN110 wt and his mutants (FIG.5) show that the pilus-2 is localized in concentrated supernatant. The typical HMW ladder is detected by anti PitB antibody only for PN110wt and PN110_pitA mutant as expected, because PN110_PI2 mutant is the negative control and PN110_PitB does not form the pilus structure without the backbone.

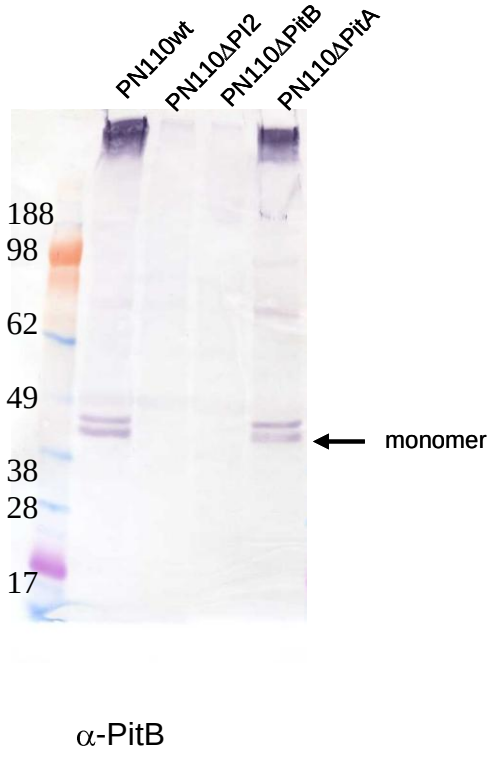
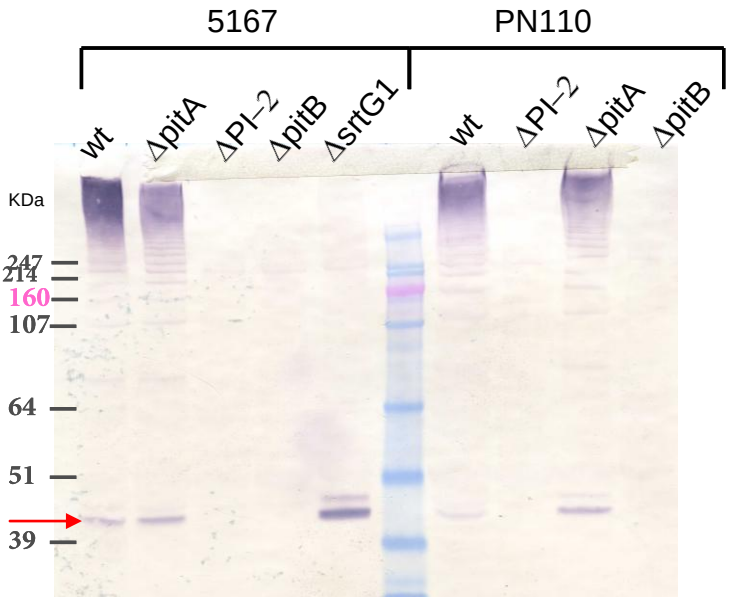


FIG.5. Localization of PitB in purified pili type 2 of *S. pneumoniae*. Western blotting performed with anti PitB on concentrated supernatants in PN110 wt and his mutants.

Given the sequence anomalies we found in *pitA*, we next sought to verify if the pilus-2 was composed only of the pilus backbone PitB or if PitA was somehow expressed and incorporated into the pilus. In order to test this hypothesis, we cloned and expressed a recombinant fragment of PitA containing the portion of *pitA* starting after the internal premature stop codon. Antibodies raised against the recombinant protein were used in Western blot analysis to probe the expression of PitA in cell wall mutanolysin extracts and concentrated supernatants. Anti-PitA antiserum (FIG. 6B) did not reveal the typical HMW ladder detected under the same conditions by the antibody anti-PitB (FIG 6A).

A)



α PitB

PitA expected MWs:

FL: 87.4 KDa

Cloned (short) after stop codon: 67.3 KDa

B)

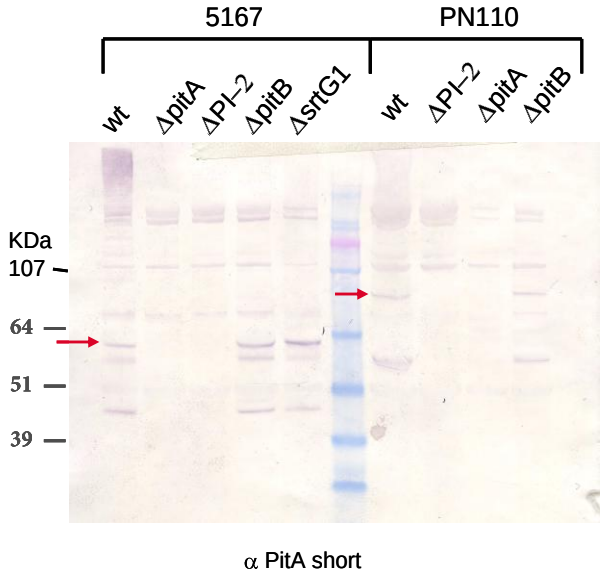


FIG.6: Western blots performed with A) anti-PitB and B) anti-PitA (short) on mutanolysin extracts in PN110 wt and 5167wt strains and their mutants.

Nonetheless, on PN110 wt and PN110ΔPitB concentrated surnatants (FiG.7A) the antibody recognized a band of about 87kDa, absent in PN110ΔPitA PN110ΔPI-2. The presence and the size of this band are compatible with the expression of a full length form of PitA. Interestingly, an analogous pattern was observed in 19FTw14 and its isogenic mutants, whereas, in the strain 5167, presenting in *pitA* sequence a frameshift in addition to the stop-codon, under the same experimental conditions the antibody detected a band of about 67 KDa. Also in this case the band size is compatible with the expression of a shorter form of PitA, containing only the C-terminal part of the protein starting after the frameshift.

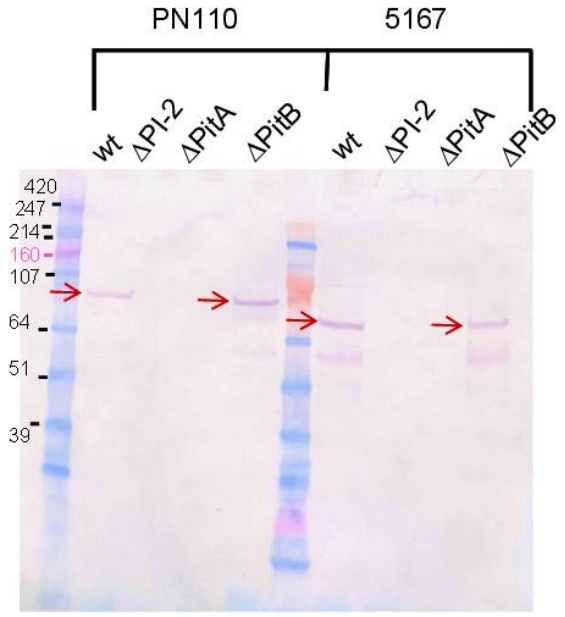
In order to demonstrate that the antibody detected band was definitely PitA, in Coomassie stained PN110 wt and PN110ΔPitB concentrated supernatants and mutanolysin extracts, a band of about 87kDa was excided, trypsin digested and analyzed by Maldi TOF mass spectrometry. This analysis, maybe because of inadequate sensitivity levels, didn't reveal the expression of PitA.

Finally, we cloned and expressed the N-terminal portion of PitA (about 15 KDa) ending before the stop codon (FIG.7B). Antibodies raised against this fragment detected by Western blotting both in PN110 strain and 5167 strain and their mutants a band of about 15 kDa corresponding to the N-terminal PitA, but did not detect the full-length form of PitA in PN110.

At this moment we cannot exclude the possibility that *pitA* is expressed through an alternative translation of the UGA stop codon located inside the open reading frames. Indeed, UGA can be translated into tryptophan at a very low frequency, and a low level of the protein could be present but undetectable by our assays. In conclusion, *srtG2* and *pitA* could be considered pseudogenes that may have lost their ability to encode functional proteins.

Pit A expected MWs:
FL : 87.4 KDa
Cloned (short) after stop codon: 67.3 KDa

A)



PitA-short

Pit A expected MWs:
FL : 87.4 KDa
N-term (before stop codon): 15.5 KDa

B)

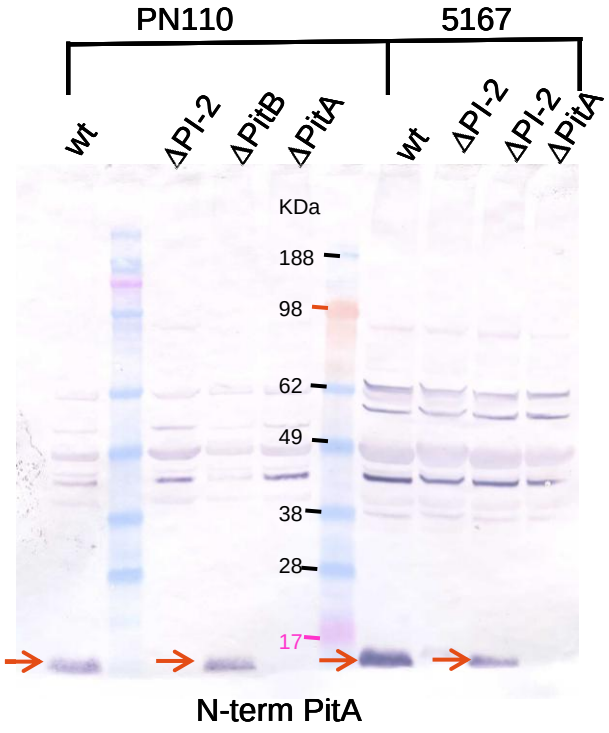
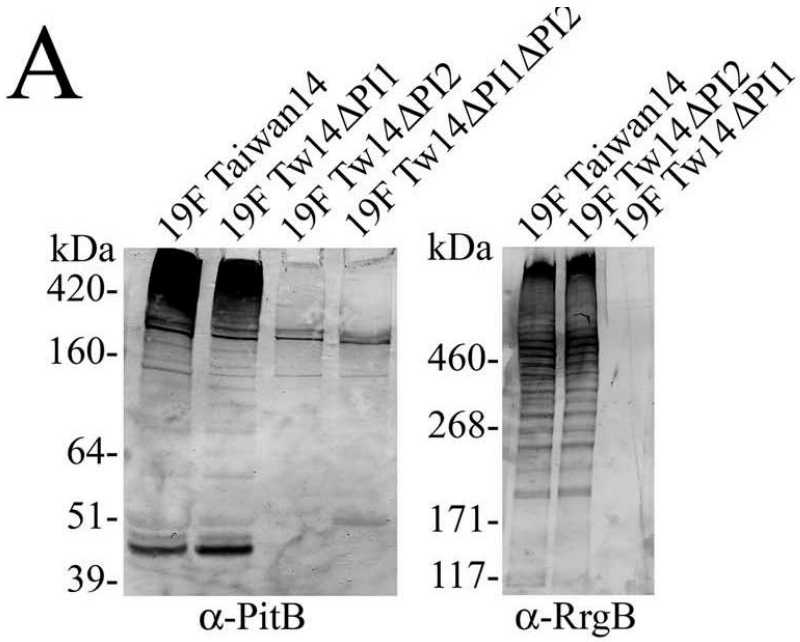


FIG. 7. Western blots performed on concentrated supernatants. Western blotting performed with A) anti-Pit A (short) and with B) anti-PitA (N-term) on concentrated supernatants in PN110 wt and 5167 wt strains and their mutants.

Interestingly, by epidemiological analysis we found that the isolates belonging to CC271 contain both PI-1 and PI-2. Through western blot and immunogold IE microscopy experiments performed with anti-PitB and anti-RrgB antibodies, we then demonstrated that the CC271 strain 19FTW14 was able to contemporarily express both pili. To verify that the assembly of the two pili is independent we generated insertional mutants on this strain by alternatively deleting PI-1, PI-2, or both. Western blot analysis and immunogold electron microscopy (Fig. 8) performed with anti-PitB and anti-RrgB antibodies demonstrated that both pili are separately assembled on the surface of 19F Taiwan-14 and that the assembly of the two pili is independent (Fig.8 C and D).



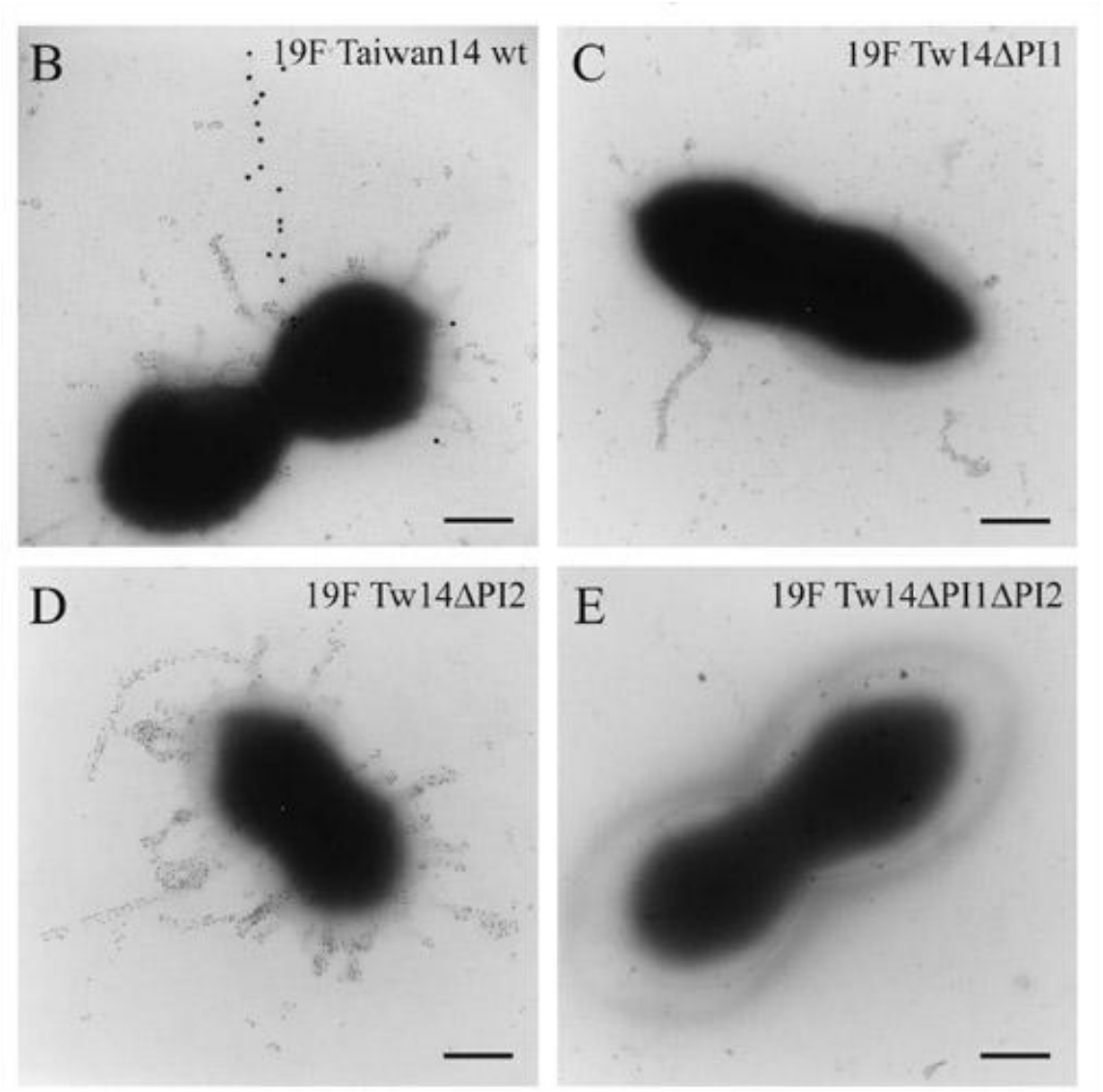
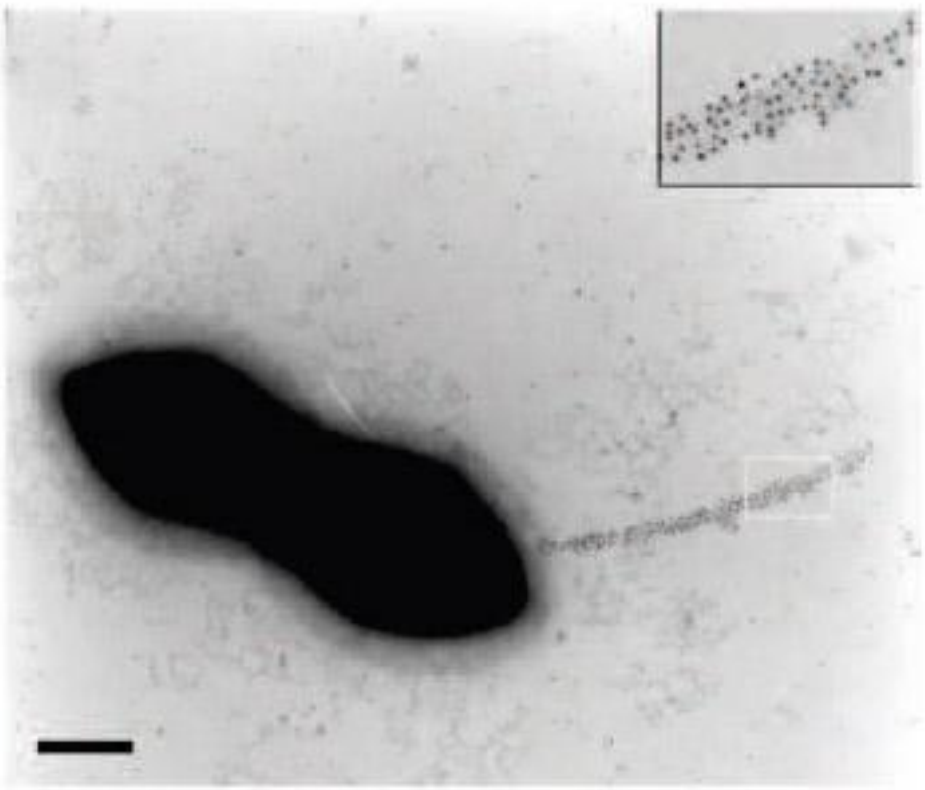


FIG. 8. *S. pneumoniae* 19F Taiwan-14 strain expresses two independent pili. (A) Western blotting performed with polyclonal anti-PitB and anti-RrgB antibodies on mutanolysin extracts of 19FTaiwan-14 wild-type and knockout isogenic mutants lacking PI-1 and/or PI-2 (19F Tw14_PI1, Tw14_PI2, and Tw14_PI1_PI2). (B and E) Double immunogold labeling performed with mouse anti-PitB (gold particle size, 20 nm) and guinea pig anti-RrgB (gold particle size, 5 nm) on 19F Taiwan-14 wild type (wt) and 19F Tw14_PI1_PI2. (C) Deletion mutant 19F Tw14_PI1 labeled with mouse anti-PitB (gold particle size, 5 nm). (D) 19F Tw14_PI2 labeled with mouse anti-RrgB (gold particle size, 5 nm). Scale bar, 0.2 nm. α, anti.

2.4 Characterization of the second pilus type

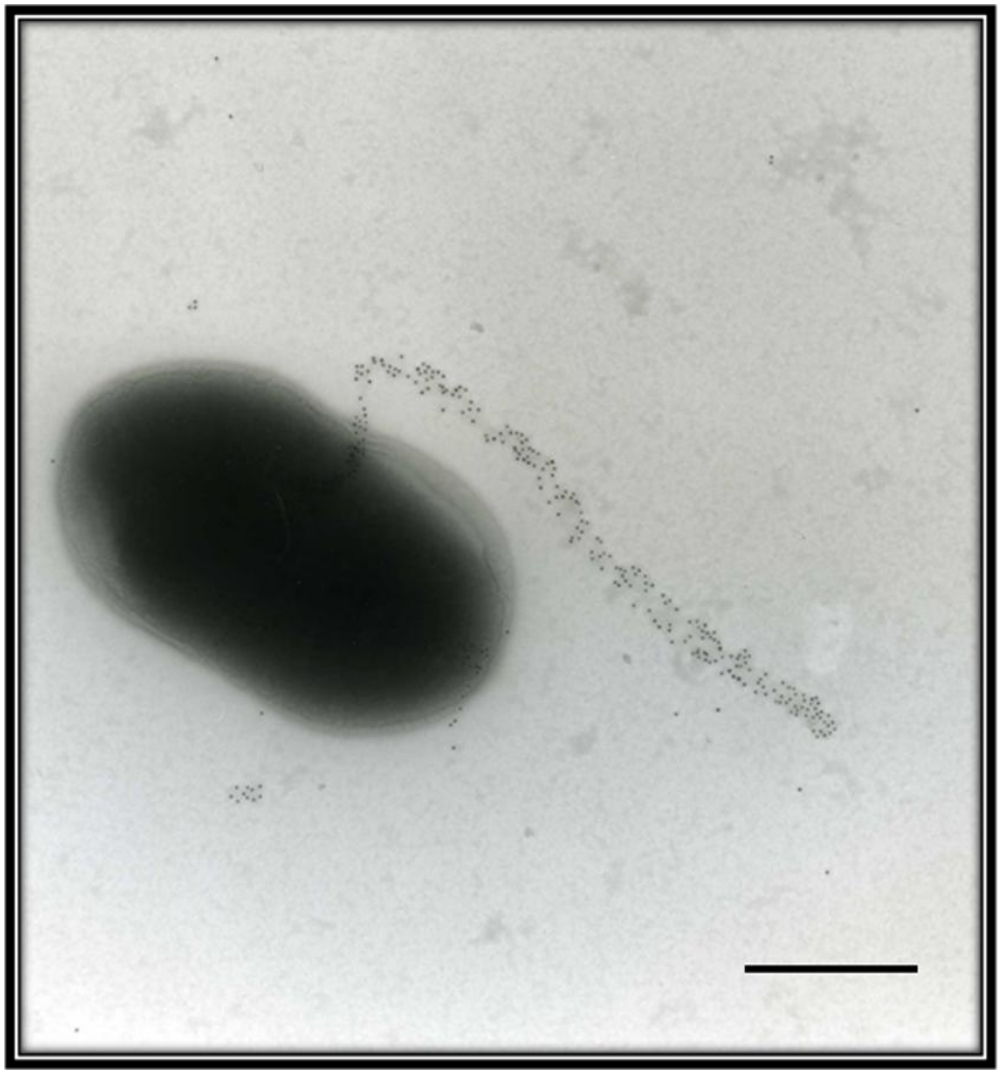
Immunogold electron microscopy, performed on wild-type serotype 1 strain PN110 with anti-PitB antibody, reproducibly revealed the presence of a single pilus copy up to 1 µm in length and 30 nm thickness extending from the bacterial surface. The pilus shaft resulted evenly decorated by 5nm gold particles bound to the anti-PitB antibody, as shown in Figure 9A. Notably, this is a peculiar characteristic of pilus-2 expression, because pilus-1, as shown in FIG. 8, is instead present in many copies per bacterium.Type1 pili appear as elongated structures up to 1 µm in length, having a compact super structure of various diameters, ranging from 40 up to 80 nm. Moreover, an analogous pilus-2 distribution/expression was detected on the surface of PN110ΔPitA bacteria, a knock out mutant of the putative ancillary protein by IEM (Figure 9B). This result strongly suggested that the PI-2 pilus shaft is composed by multiple copies of the same PitB molecules and that PitA is neither necessary for pilus formation nor for pilus-2 expression regulation.

A)



PN110wt, α-PitB

B)

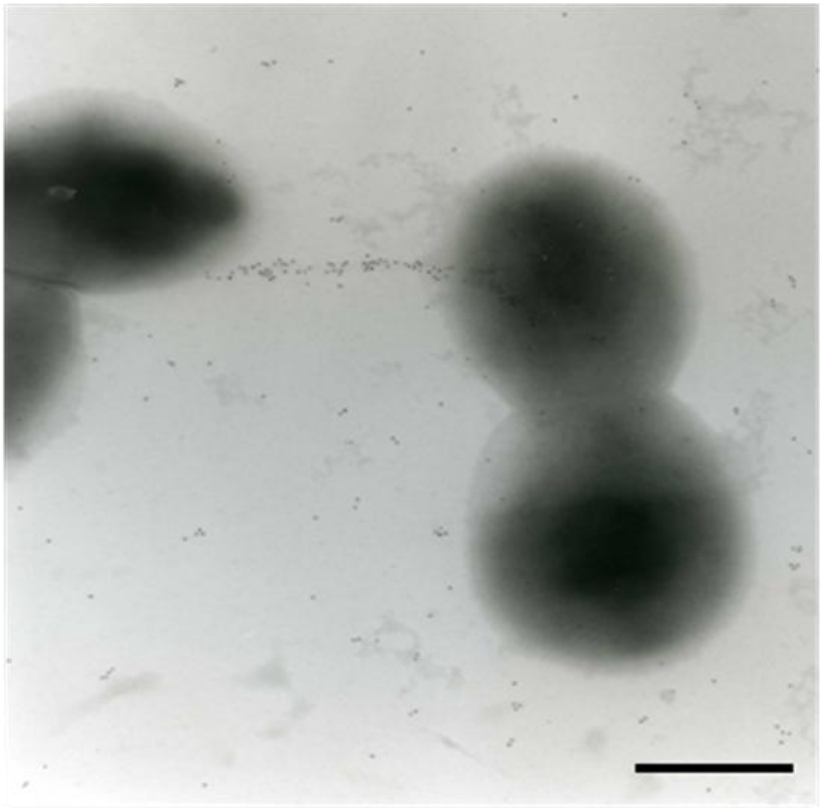


PN110ΔPitA, α-PitB

FIG. 9 A) Immunogold Electron Microscopy localization of PitB in pili of *S. pneumoniae* PN110 whole cells. Inset shows an enlarged portion of the pilus. α, anti. Scale bar 100nm B) Immunogold labeling with anti-PitB antibodies of whole-cell PN110 deletion mutant, PN110ΔpitA. Bacteria were charged on Formvar carbon grids and immunogold decorated with mouse anti-PitB (gold particle size, 5 nm). Scale bar 100nm.

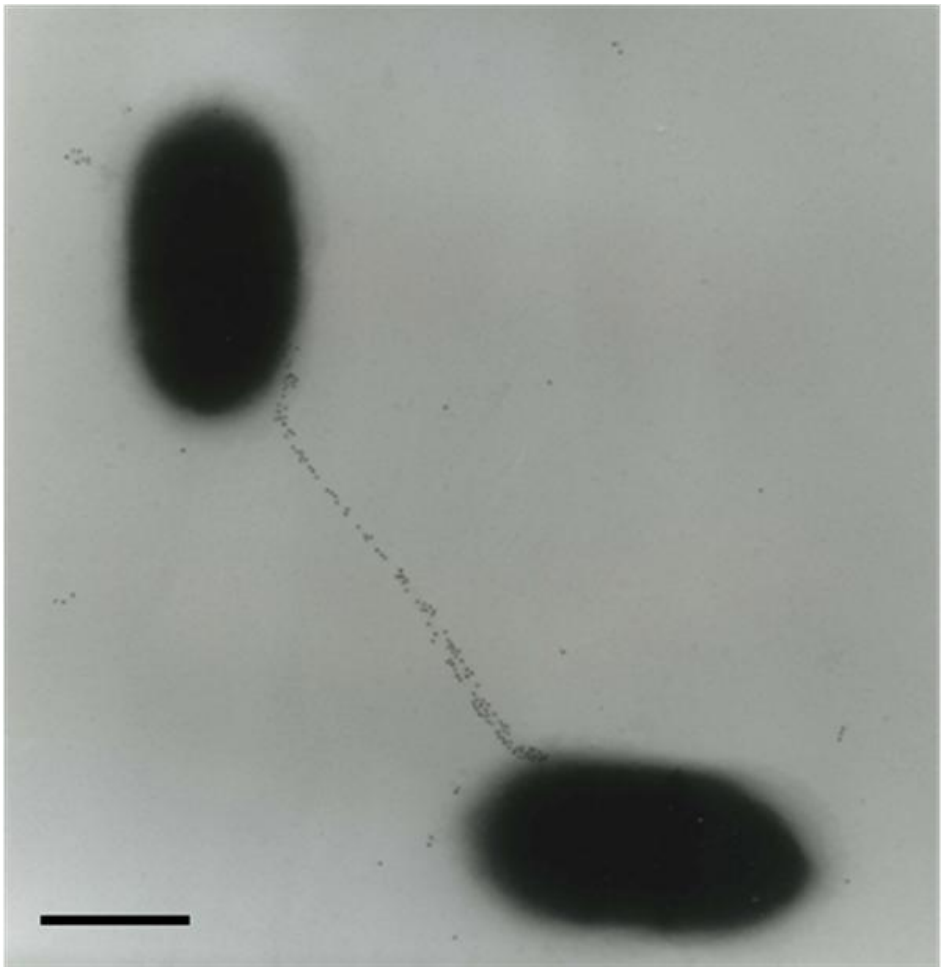
Interestingly, when PN110wt and PN110ΔpitA bacteria (Fig.10), were grown until they reached the late growth phase, some of the bacteria present in the preparation appeared to be connected through pilus-2. These typical situations were noticed growing the pneumococci 13 hours on THYE plates, resembling in this way the late growth phase, when the bacteria are particularly stressed. The connecting pilus showed to have the same structural characteristics and the dimensions of the single pilus expressed on the bacterial surface during exponential phase growth.

A)



PN110 wt, α-PitB

B)



PN110ΔPitA, α-PitB

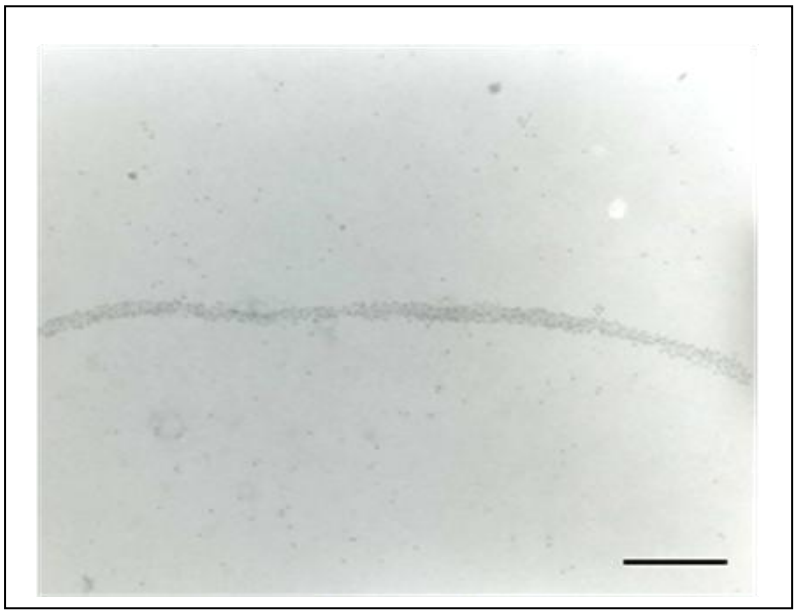
FIG. 10. Immunogold labeling with anti-PitB antibodies of whole-cell PN110 A) While type. B) Deletion mutant, PN110 ΔPitA.Bacteria were charged on Formvar carbon grids and immunogold decorated with mouse anti-PitB (gold particle size, 5 nm). Scale bar 100nm.

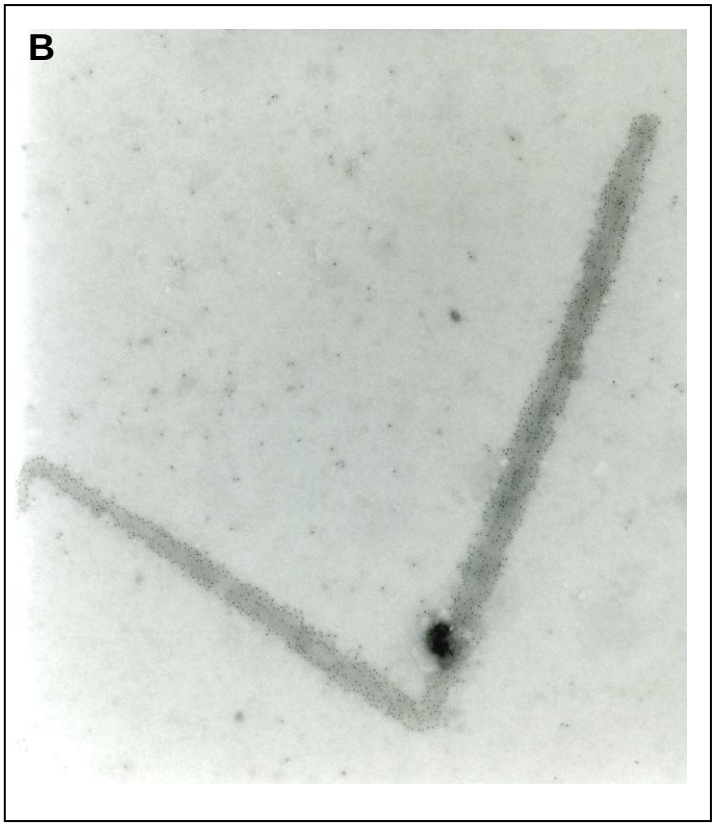
2.5 The second pilus type structure

In order to get some insight on the structure on the PI-2 pilus we performed both IEM and negative stain TEM on purified native pili preparation.

Purified native pili (FIG11) were obtained from *PN110 wt* strain grown in THYE broth until they reached O.D. 0.6-0.9. ($0.6<OD_{600}<0.9$). At this optical density value, bacteria were centrifuged and the cleared supernatants concentrated about 50 times (as described in Materials and Methods).

A




B

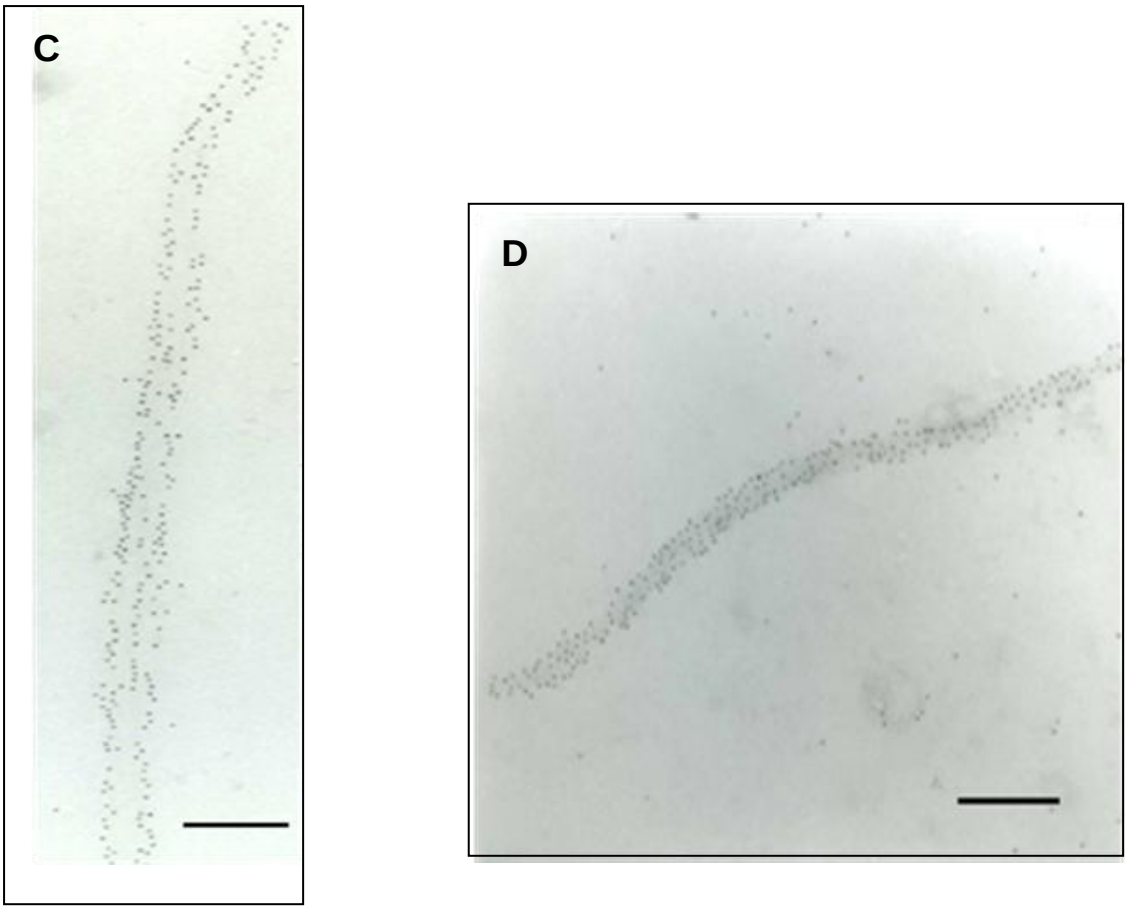


FIG. 11. Localization of PitB in purified pili type 2 of *S.Pneumoniae*. A) Western blotting performed with anti PitB on concentrated supernatants in *PN110 wt* and his mutants.
B to E) Immunogold labeling with anti-PitB antibodies of purified second pilus type. Pili purified from different cultures at the same optical density (OD600=0.6) but different magnification B)-C) of 21K D) of 28.5 K E) of 39K. Scale bar 100nm.

The presence of purified pili in the obtained supernatants were checked by Immunogold labeling using polyclonal antibodies against PitB, As secondary antibody anti mouse bound to 5nm gold size particles, was used.

Native type 2 purified pili appeared as long as the type 1purified pili, but thicker with diameters ranging between 40 and 80 nm. Interestingly, the diameter and the length

of pili were strictly correlated to the growth phase. At OD_{600}=0.2, at the beginning of the exponential phase growth, pilus was very short (not longer than 10nm) or fragmented in few small parts (FIG.12A); at OD_{600}=0.6 and OD_{600}=0.9 pilus became longer and thicker (FIG.12B and C) reaching the final dimensions already described.

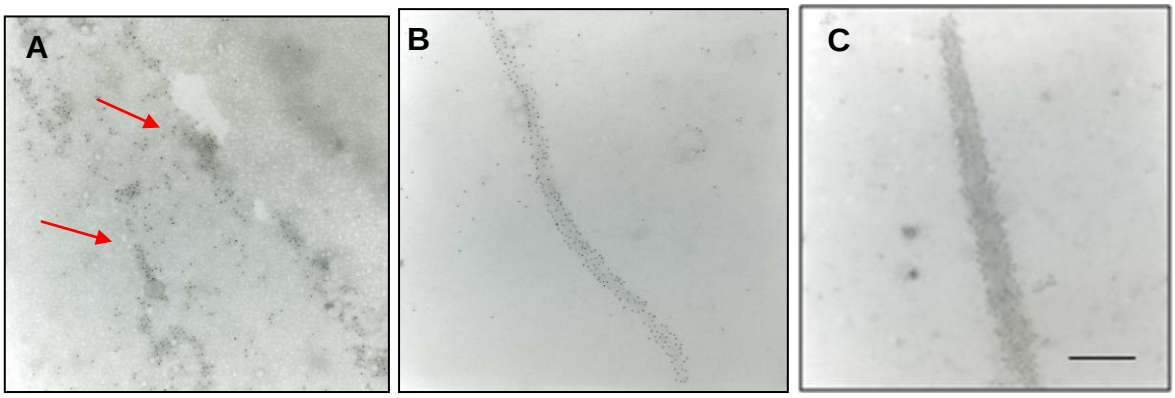
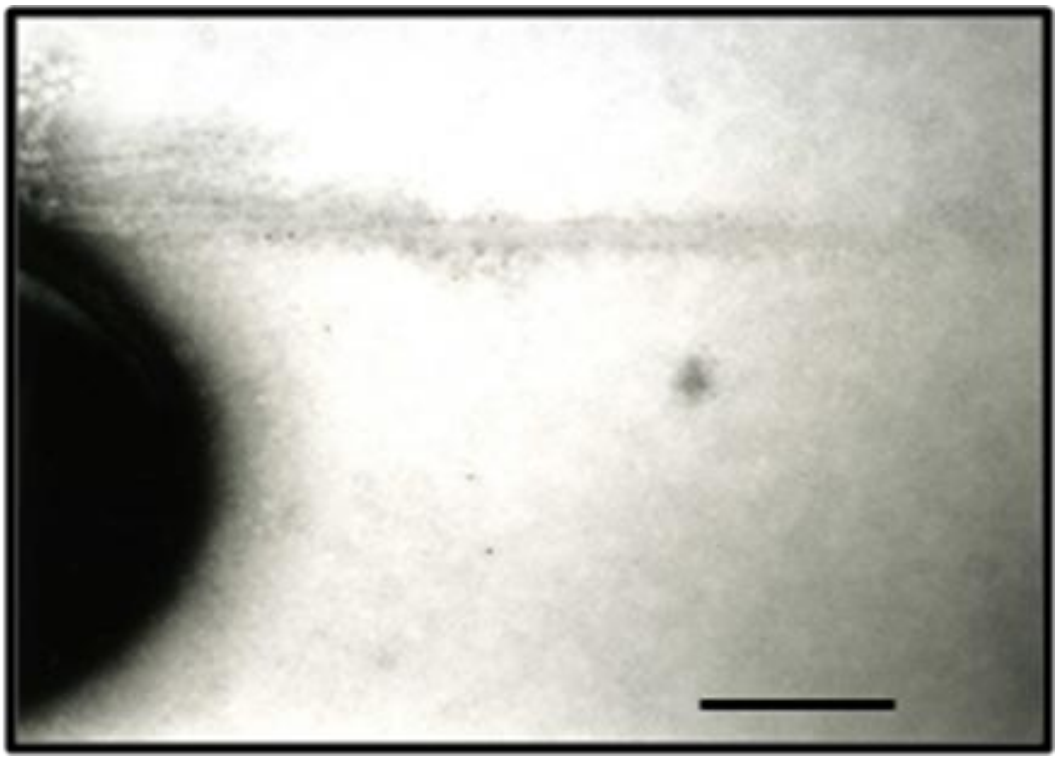


FIG. 12. Purified pilus type 2. Immunogold labeling localization of PitB in purified pili of *S. pneumoniae* on different growth phases of the same culture. A) OD600 = 0.2, B) OD600 = 0.6, C) OD600 = 0.9. Magnification of 39 K. Scale bar 100nm.

2.6 Preliminary structural analysis of pilus-2

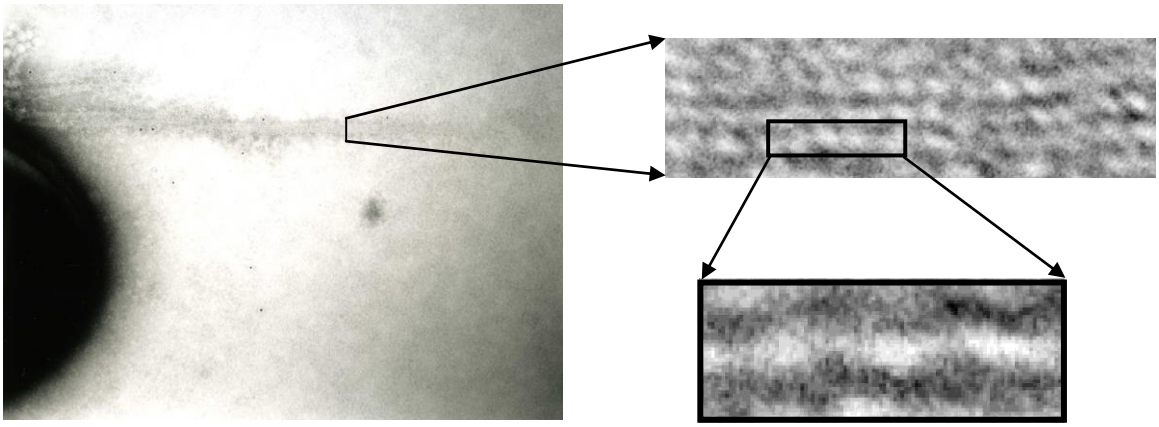
The detailed composition of pili was investigated by single IEM with antibodies raised against the backbone protein and the ancillary protein. Single IEM performed on bacteria with one antibody at the time revealed that the pilus is composed by the only backbone protein that was evenly distributed along the entire pilus polymer. PN110wt bacteria when gentle labeled with lower concentrations of both primary and secondary antibodies showed on their surface the individual pili composed of several filaments (Figure 13 panel A and B) with only few gold particles decorating the pilus. The average number of filaments forming the single pilus was ranging between 5 and 10. We observed that all the filaments forming the pilus were running parallel to each other keeping a distance of less than 1 nm between them (Figure 13 panel C). Interestingly, the single filament when observed at higher magnification showed to be composed by a series of molecules arranged in a head-to-tail organization generating “necklace pearl” like filaments. The single subunit showing a bean shape, had dimensions of 3.5 nm in length and 2 nm in width (Figure 13 panel D).

A)



B)

C)



D)

FIG.13. Structure as filaments of pilus2. Pilus 2 structural analysis A)-B) light immunogold labeling of PN110 wt bacteria with antibody α-PitB (5nm gold size). Scale bar 100nm C) Inset of a pilus 2 section showing the parallel filaments. D) Inset of the pilus2 section showing the “necklace pearl” organization of the single PitB backbone proteins along the filament.

Subsequently, the density profile of a single subunit was generated indicating that the subunit is rather a compact structure (Figure 14).

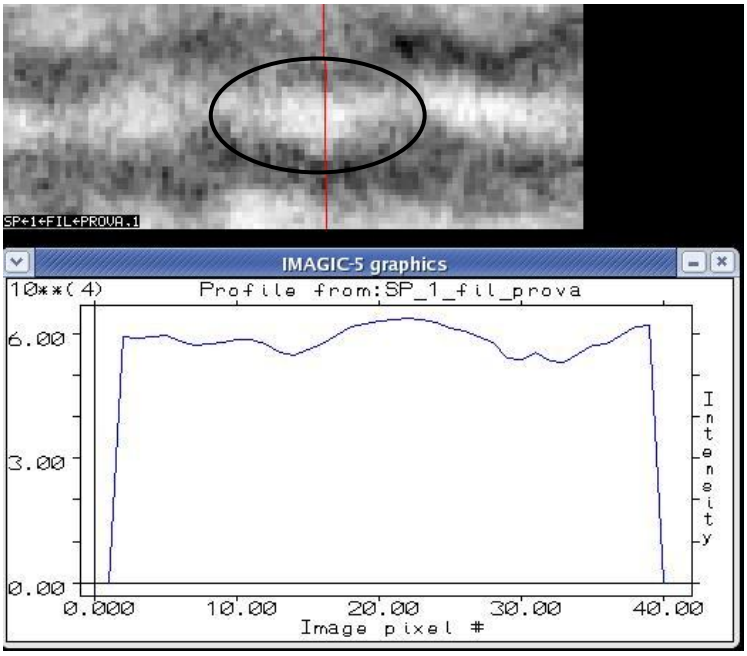


Fig .14. Density profile of a single “necklace pearl” subunit identified in the pilus-2 fiber. The profile shows clearly the compactness of the protein. Dimension of the subunit are corresponding to a protein with a MW of 35-40 kDa.

2.7 Materials and Methods

Strain collection. A total of 305 isolates collected worldwide (Table 1) have been analyzed for the presence of PI-2. In detail, these isolates include the following: 26 clones from the Pneumococcal Molecular Epidemiological Network (PMEN) collection (24); 50 invasive pneumococcal isolates from the Centers for Disease Control and Prevention (CDC; Atlanta, GA) (6); 55 clinical isolates (28 carriage and 27 meningitis isolates) from Salvador, Brazil; 15 and 36 invasive clinical isolates from Ghana and Bangladesh, respectively (17); 40 clinical isolates from Italy; 5 invasive clinical isolates from Kenya; 8 clinical isolates from Sweden; 43 nasopharyngeal isolates from Norway (40a); and 11 laboratory strains from the Novartis collection and 13 from the University of Alabama.
Additionally, data about PI-2 presence have been extrapolated for three isolates from sequence data available at the Sanger website (www.sanger.ac.uk).

PI-2 detection and sequencing. The INV104 (genomic sequence available at www.sanger.ac.uk) PI-2 nucleotide sequence (*srtG2-pitA*) was analyzed, and a set of 28 oligonucleotide primers was designed: 24 were specific for regions inside the islet, while 2 annealed in two conserved genes (corresponding to SP1008-SP1009 of the *S*. *pneumoniae* TIGR4 genome sequence) that flank the islet. The set of primers was used to detect both the presence and location of PI-2 within all the isolates in the collection, as well as for sequence analysis of the entire locus. Briefly, the genomic location of the islet was determined by simultaneously assessing four PCR amplifications as follows: the first primer pairs (1008for and 1009rev) matched in regions flanking the operon (when PI-2 was absent, a lower fragment size was detectable); the second and third amplifications used the primer pairs 1008for-INTrev and INTfor-1009rev, where
INTfor and INTrev annealed within the islet (fragments detectable only when

the islet was present and inserted in the genomic region comprised between SP1008-SP1009); and the fourth amplified conserved regions within the islet (amplification detected whenever the islet was present in the genome).

Generation of *S. pneumoniae* mutants. Serotype 1 PN110 (Istituto Superiore di Sanita [ISS] collection) and serotype 19F Taiwan-14 (PMEN) isogenic mutants described in Table 3 were made by PCR-based overlap extension. Briefly, fragments of approximately 500 bp upstream and downstream of the target gene were amplified by PCR and spliced to an antibiotic cassette (kanamycin or erythromycin); the PCR fragments were then cloned into pGEMt (Promega) and transformed in the appropriate *S. pneumoniae* strain by conventional methods (3). To select the bacteria in which the target gene was replaced with the antibiotic cassette, bacteria were plated on blood-agar plates with erythromycin (1 _g/ml) or kanamycin (500 _g/ml). Mutants were confirmed by PCR, sequencing and Western blot analysis.

Strain[a]	Relevant characteristic(s)
PN110 wt	Serotype 1 PN110 strain
PN110ΔPI2	PI-2::Kan
PN110Δ*pitA*	*pitA*::Kan
PN110Δ*sipA*	*sipA*::Kan
PN110Δ*pitB*	*pitB*::Kan
PN110Δ*srtG1*	*srtG1*::Kan
PN110Δ*srtG2*	*srtG2*::Kan
19F Taiwan-14 wt	Serotype 19F Taiwan-14 strain (PMEN clone)
19F Tw14ΔPI1	*rlrA*::Erm
19F Tw14ΔPI2	PI-2::Kan
19F Tw14ΔPI1ΔPI2	*rlrA*::Erm PI-2::Kan

[a] wt, wild type.

TABLE 4. Definition of isogenic knock-out mutants generated on PN110 and 19F Taiwan14 in this study

Biochemical analysis of *S. pneumoniae* pili and immunoblotting. Mutanolysin extracts and concentrated surnatants for pilus polymerization analysis were obtained as previously described. Samples were loaded onto 4 to 12% Bis Tris or 3 to

8%NuPage Tris gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were then immunoblotted with serum against the pneumococcal antigens and with secondary antibodies conjugated to alkaline phosphatase (Promega). Membranes were developed according to manufacturer's instructions.

Flow cytometry. Bacteria were grown in Todd-Hewitt broth containing yeast extract to exponential phase (optical density at 600 nm [OD600] of 0.2), fixed with 2% paraformaldehyde, and then treated with mouse antisera raised against PitB recombinant protein (anti-PitB antibody, dilution 1:100). After samples were labeled with a fluorescein isothiocyanate-conjugated secondary antibody (Jackson Laboratories), bacterial staining was analyzed using a FACScan flow cytometer (Becton Dickinson). Sera from mice immunized with phosphate-buffered saline (PBS) plus adjuvant were used as negative controls.

Statistical analyses. For adherence assays, data were analyzed by a Student's *t* test.

Nucleotide sequence accession numbers. The following GenBank accession numbers were assigned to *S. pneumoniae* PI-2: serotype 7F strain 32_14, EU311532; serotype 19F strain 5167-99, EU311533; serotype 2 strain 31620, EU311534; serotype 1 strain PN110, EU311535; serotype 19F strain pgx1416, EU311536; serotype 19F strain SP231, EU311537; serotype 1 strain SPPD, EU311538; and serotype 19F strain Taiwan-14, EU311539.

Sample preparation of purified pilus type 2. Bacteria of PN110wt strain were grown overnight on TS agar (13h)**.** The main culture (100 ml) was grown in filtered Todd-Hewitt broth containing yeast extract to different exponential phases (Optical density at 600 nm [OD600] of 0.2/0.6/0.9. Bacteria were centrifuged 20 min x 4000 g at 4°C and the surnatant concentrated with Amicon ultra-15 Centrifugal filter Devices (100k): 20 min x 4000 g at 4°C at different Optical Density. (OD=0. 2/0.6/0.9) The

samples were storied (aliquots at 4°C, - 20°C, - 80°C) for IEM labeling and for structural studies.

Immunogold labeling electron microscopy and structural analysis. Bacteria were grown overnight on blood-agar plates, resuspended in PBS, charged onto Formvar-coated nickel grids, let stand for 5 minutes and subsequently fixed with 2% paraformaldehyde and 1XPBS before being labeled with 1:10 (dilutions of polyclonal α-PitB in blocking buffer (1% BSA, in PBS) (when gentle IEM was performed a dilution of 1:200 of primary antibody was used)Samples were then washed with blocking buffer and subsequently incubated with a 1:20 dilution of a secondary goat α-mouse IgG (5nm) conjugated to gold particle (BBInternational) (when gentle IEM was performed a dilution of 1:50 of secondary antibody was used). Finally, samples were washed with 5 drops of distilled water, and stained with 1% phosphotungstic acid (PTA), before analysis in a CM10 transmission electron microscope (TEM, Philips Electronic Instruments, Inc) operating at 80 kVAll images were collected at a nominal magnification of 50000 X, on Kodak SO163 film. Micrographs where checked for astigmatism and drift on an optical diffractometer prior to digitisation. Pili segments were picked manually from digitized images using the command “helixboxer” from the software EMAN (Ludtke SJ, et al 1999)

CHAPTER 3

Discussion

3.1 Identification of second pilus type sequence

Pili are important key players in bacterial pathogenesis and infection. Up to now functional and structural informations of native pilus in Streptococcus pneumoniae bacteria is available only for pilus-1; this analysis has not been yet performed on pilus-2.

In this study, we report the identification of a second PI, PI-2, in *S. pneumoniae*, a major cause of disease in children. Pili or fimbriae are extracellular organelles located on the surface of bacteria. In gram-negative pathogens, pili are important virulence factors involved in conjugation between bacteria, adhesion to the host, motility, and transfer of effector molecules. In gram-positive organisms, pili are not as well studied. However, reports indicate that *C*. *diphtheriae*, as well as *Streptococcus* spp. and *Actinomyces* spp. have elaborate pili composed of LPXTG-type protein subunits. These subunits are covalently linked to one another by sortase-mediated transpeptidation reactions. Lack of pilus-specific sortases abolishes the polymerization of the pilus.

In *S. pneumoniae,* PI-2 is similar to previously described gram-positive pili in its global genetic organization and in its sequence homology with the FCT-3 pilus from *S. pyogenes*. Indeed, the islet contains five genes coding for two putative sortases (*srtG1* and *srtG2*), a signal peptidase-related protein (*sipA*), and two LPXTG-type surface-anchored proteins (*pitB* and *pitA*). Interestingly, through BLAST iterative searches with the PI-2 proteins, we identified a group of conserved hypothetical proteins, organized as a locus in the genome of the human intestinal gram-positive bacterium *Ruminococcus gnavus*; the homology between *S. pneumoniae* backbone protein PitB and *R. gnavus* was 27%, while for SipA and SrtG1 the similarity increased to 43% and 41%, respectively. This finding suggests that horizontal transfer may occur between bacteria of different genera and that the human commensal *Ruminococcus* spp. could also encode pilus structures.

The results presented in this work demonstrate that PitB is the pilus-2 backbone subunit, as already suggested by the sequence analysis. In fact, deletion of the other CWSS-containing protein PitA does not affect pilus polymerization. Electron

Microscopy observations show elongated structures that resemble the previously described RrgB pilus filaments although many fewer filaments per bacterium were present (Fig.9). In the case of the isolate 19F Taiwan-14, it is evident that the PI-1 pili cover most of the surface of the pneumococcus (Fig.8B and D), while PI-2 pili are found in single copies and extend further from the cell (Fig.8B and C). The diameter of PI-2 is approximately between 60-100 nm. The different morphology of pilus-2 with respect to pilus-1 let us think that it could play a different role during the infection.

3.2 Characterization of each PI-2 subunits

Polymerization of PitB requires sortase SrtG1 and the LepA-homologue SipA. This latter result confirms the importance of the signal peptidase-like protein and corroborates its hypothesized role as a chaperone in pilus polymerization, recently shown in *S. pyogenes*. In addition, protein sequence alignment performed between SipA and other known and hypothetical signal peptidases shows the lack in SipA of the two conserved residues, serine and lysine, required for peptidase activity. An analogous situation was found in SipA1 and SipA2 of *S. pyogenes*. We next investigated the role of the hypothetical ancillary protein encoding gene *pitA* and the putative sortase gene *srtG2*; we found that *pitA* contains a stop codon in all nine sequences analyzed, that *srtG2* contains a frameshift in all but one isolate, and that the lack of these genes does not affect the pilus assembly. These observations suggest that the PI-2 pilus may be composed of only the pilus backbone PitB. To verify this possibility, we raised antibodies against a portion of the PitA starting after the internal stop codon. Western blot analysis of cell wall mutanolysin extracts with anti-PitA antiserum did not reveal the typical HMW ladder or the monomeric protein. Furthermore, mass spectrometric analysis of the mutanolysin extracts was not able to detect the PitA protein (data not shown). At this moment we cannot exclude the possibility that *pitA* is expressed through an alternative translation of the UGA stop codon located inside the open reading frames. Indeed, UGA can be translated into tryptophan at a very low frequency, and a low level of the protein could be present but

undetectable by our assays. Therefore, *srtG2* and *pita* could be considered pseudogenes that may have lost their ability to encode functional proteins.
Moreover, the low distribution of PI-2 among clinical isolate strains (16%), the low level of PI-2 expression in all the isolates tested, and the presence of frameshift mutations and stop codons, taken together, lead to the hypothesis that this second PI may not be required but could be an additional factor important for survival in the host.

3.3 Structural organization of the second pilus type

Our structural approach consisted in both obtaining whole bacteria and native purified pili from a pathogenic strain of *S. pneumoniae* to study the pilus structure. Particular emphasis was drowning on the overall structural principle of the pilus and the possible role of the individual structural and non structural proteins. The bacteria were analyzed by negative stain EM and IEM, showing that the bacteria express on their surface only one single elongated, flexible pilus-like appendage up to 1 µm long. Interestingly, we observed that the pilus doesn't have any specific location on the bacterial surface. Moreover the pilus shows, at high magnification observation, that is composed by several copies of the same elongated structure: the filament. Therefore pilus can be distinguishable into different classes by their diameter (ranging from 40 nm up to 80 nm) and number of filaments. Structural analysis based on IEM data revealed they are organized in superstructure made by several copies of the same type of filament running parallel to each other. The observed range in pilus diameters could either reflect a difference in the degree of packaging of the identical filament into the pilus superstructure or a higher number of filaments composing the larger pilus. The filaments themselves are organized to form a "necklace pearl" compact superstructure where the single pearls are corresponding to the backbone subunits (FIG16). The dimensions of each single subunit correspond to a molecular mass of 35-40 KDa giving a good indication that the filament is made by the only backbone protein (which has a molecular mass of 37 KDa).

Several experiments of bacterial growth show that bacteria produce the second pilus type after 12-13 hours. This means that bacteria start to produce plius2 during the late exponential growth phase when usually they are stressed.

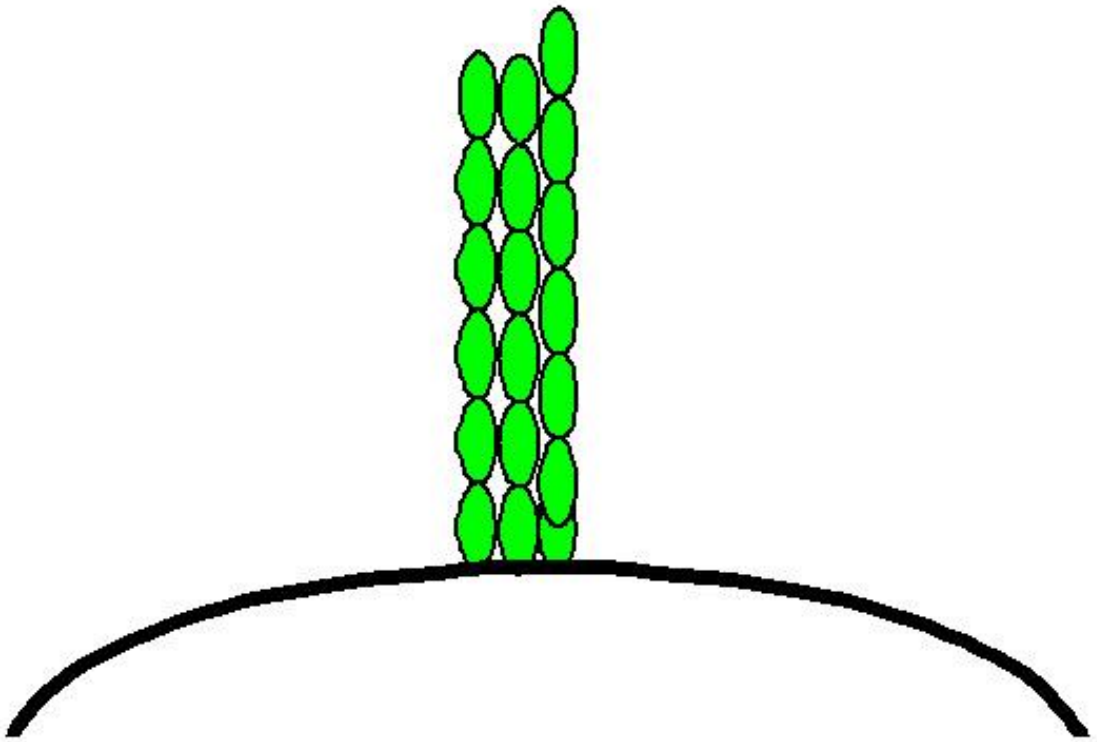


Fig.16. Model of pilus-2 structure.

3.4 Possible function of pilus-2 and its structural model

To date, all pili of Gram-positive bacteria have at least one functional ancillary protein; our group of researchers has demonstrated that ancillary proteins play a major role in adhesion (Materials and Methods). We provide evidence that this newly identified pilus favors adherence to the host cells (Bagnoli et al, J. Bacteriol. 2008). Interestingly, PI-2-mediated adherence appears to rely solely on the pilus backbone protein. Indeed, study of isogenic mutants of the islet genes has shown that expression of *pitB* is strictly required for adhesion to respiratory cells, since the PN110Δ*pitB* strain is completely impaired, as is the PN110ΔPI2 strain, in this property. On the other hand, deletion of *srtG1* did not result in the complete loss of binding capacity. PN110_*srtG1* may still express and translocate a monomeric form of PitB to the bacterial surface. Therefore, adhesion mediated by a monomeric form of PitB may explain the adhesive properties of the sortase mutant. In this regard, we evaluated adherence properties of the PitB purified protein by immunofluorescence microscopy on adherent A549 cells and by FACS analysis. Both assays showed low binding capacity of the protein, confirming our expectations for a pilus backbone subunit. However, experiments on adherent cells show a diffuse binding pattern, which suggests that PitB may recognize a cellular protein expressed at low levels. We believe that the difference in adhesion observed with the two approaches may be due to the redistribution of proteins recognized by the pilus backbone protein (PitB) over the cell surface upon detachment from the support. Given that the polymerized pilus is able to mediate stronger binding, it is likely that both the sum of the binding of the PitB subunits and the structural conformation of the pilus are important for its adhesive mechanism. However, this novel pilus is not as effective in mediating attachment to host cells as the *rlrA* pilus of *S. pneumoniae* (data not shown), and this phenomenon may be explained by the lack of a detectable expression of ancillary subunits.

Even if the backbone PitB is involved in adherence to host cells, some typical characteristics of pilus-2 let us suppose that pilus2 is more involved in conjugation. Dipper studies of the structure of pilus-2 show filaments similar of those found in

E.coli, the typical Gram-negative bacterium (Grossman H. et al, J. Bacteriol., 1990). The parallel filaments organization into the pilus-2 reminds the microtubule organization observed in some Gram-negative bacteria. In this latter case the filaments are running parallel to each other forming a hallow tube that could be used as a conjugative structure necessary to transfer genetic material from one bacteria to a second one. Three important observations could be extrapolated from our work: i) Pilus-2 is present in only one single copy per bacteria , ii) PN110 bacteria could be connected through the pilus protruding from one of the coupled bacteria; iii) the dimensions and the organization of the pilus is very similar to those of the Gram-negative microtubules. Those observations could indicate that the pilus-2 could play a role in the genetic transfer mechanism instead of having an adhesion activity. The preliminary model generated by our work is the following (see Fig.16): where the single copy of the pilus shows to have no specific location and is made by several copies of filaments running parallel and with each filament resulting as a polymer made by the same backbone subunit (green spheres) organized in a head-to-tail arrangement. Additional structural, functional and biochemical analysis will be performed in order to clarify the pilus-2 role in bacterial life cycle.

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