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Pneumococcus: the sugar-coated bacteria

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Summary. The study of *Streptococcus pneumoniae* (the pneumococcus) had been a central issue in medicine for many decades until the use of antibiotics became generalized. Many fundamental contributions to the history of microbiology should credit this bacterium: the capsular precipitin reaction, the major role this reaction plays in the development of immunology through the identification of polysaccharides as antigens, and, mainly, the demonstration, by genetic transformation, that genes are composed of DNA—the finding from the study of bacteria that has had the greatest impact on biology. Currently, pneumococcus is the most common etiologic agent in acute otitis media, sinusitis, and pneumonia requiring the hospitalization of adults. Moreover, meningitis is the leading cause of death among children in developing countries. Here I discuss the contributions that led to the explosion of knowledge about pneumococcus and also report some of the contributions of our group to the understanding of the molecular basis of three important virulence factors: lytic enzymes, pneumococcal phages, and the genes coding for capsular polysaccharides. [**Int Microbiol** 2006; 9(3):179-190]

Key words: *Streptococcus pneumoniae* · capsular polysaccharide · cell wall hydrolases · bacteriophage · virulence factors

Introduction

Until the 1940s, *Streptococcus pneumoniae* (the pneumococcus) was a dangerous human killer. In fact, it was the leading cause of death and was nicknamed "the captain of the knight of death" because it caused more health problems than cardiovascular disease and cancer together [70]. In 1944, Avery, McLeod, and McCarty [5], using the major virulence factor of pneumococcus (the capsular polysaccharide) as the phenotypic marker, were able to demonstrate that genes were made of DNA. The current importance of this historic bacterium comes from concern over pneumococcal disease caused by multidrug-resistant strains. Infectious diseases are the third leading cause of death in the United States and the leading cause of morbidity worldwide, with pneumococcus being the main cause of pneumonia, meningitis, and bloodstream infections in the elderly, the young, and immuno-compro-

mised individuals [54, 58]. In addition, pneumococcus is the major cause of middle-ear infections in children. The interest of many groups of scientists to provide answers to questions concerning the biology of pneumococcus and its ability to cause disease is justified by the global importance of *S. pneumoniae* as a cause of illness, sequelae, and death, and because the spread of drug resistance is undermining our ability to treat pneumococcal infections [33]. New diagnostic tests and the development of improved vaccines are needed to combat the threat from multiple drug resistance, as is research on the control of DNA transfer in nature and the control of capsule production. Existing vaccines have only limited efficacy, and attempts to combat pneumococcal infections through a more generalized use of antibiotics seems unrealistic in the long-term because of the genetic plasticity of this bacterium, which results in a shift in capsular type or in the rapid spread of antibiotic-resistant isolates and the appearance of novel antibiotic resistance 'determi-

nants'. Recently, phages and phage products have been proposed as an alternative (or complement) to available antibiotics.

This review provides an updated insight into some aspects of the historical importance of pneumococcus and its current clinical importance. It also summarizes current knowledge regarding the molecular biology of the major genetic traits that play a fundamental role in pneumococcal microbiology and the development of disease, as well as strategies to prevent and treat diseases caused by this dangerous human pathogen.

From Pasteur and Sternberg (1881) to 1950.

The pneumococcus is a normal component of the microbiota of the human respiratory tract and a major gram-positive human pathogen. It has had a long history, one that is integrally connected to the history of several fields of biology, including microbiology and molecular biology. At the beginning of the twentieth century, pneumococcal pneumonia was the leading cause of death and, as quoted by Maclyn McCarty (1911–2005), research directed against this specific medical problem also resulted in a breakthrough in molecular biology [46,47]. In 1880, George Sternberg (1838–1915) inoculated rabbits with his own saliva [61] while Louis Pasteur (1822–1895) used the saliva of a child that had died from rabies [59]. Their experiments resulted in the isolation of a lanceolatus micrococcus, later known as *Diplococcus pneumoniae*. The availability of new biochemical and molecular techniques for taxonomic identification led to the classification of this bacterium within the genus *Streptococcus*, as *Streptococcus pneumoniae*. Sternberg and Pasteur also recognized the presence of a capsule surrounding the diplococcal form of this microorganism. In 1882, Friedländer identified the pneumococcus as the major cause of human lobar pneumonia [21], and in 1884, Gram developed his now famous stain technique to facilitate identification of pneumococcus in histological sections of the lungs [25].

Credit must also be given to the remarkable contribution of Fred Neufeld (1869–1945) in the identification of characteristics that differentiate pneumococcus from other bacteria. In 1900, he described the bile solubility test [55] and, in 1902, the Quellung reaction [56]. The bile solubility test is based upon triggering of the uncontrolled activity of the major pneumococcal lytic enzyme (the amidase LytA) in response to bile treatment, while Quellung ("swelling" in German) refers to the refractive property of the pneumococcal capsule when exposed to homologous antibodies, the so-called capsular precipitin reaction. Originally, this reaction was erroneously used to indicate a "swelling" of the capsular polysaccharide that involved pneumococcus. This technique was introduced in 1931 by Neufeld and R. Etinger-Tulczynska as the preferred method for typing *S. pneumoniae* [57]. Interestingly, pneumococcus has also played a historical role in the development of immunology, when Alphonse R. Dochez (1882–1964) and Oswald T. Avery (1877–1955) reported that the specific soluble substances of pneumococcus were polysaccharides with antigenic properties similar to those previously ascribed, exclusively, to proteins [14].

As cited above, the remarkable clinical importance of pneumococcus attracted many researchers to study the peculiarities of *S. pneumoniae* and they became soon aware of the crucial role played by capsular polysaccharide in the virulence of a given pneumococcal type, of which 90 have been identified to date [31]. The recognition that loss of capsulation by pneumococcus resulted in a loss of virulence led Avery and René Dubos (1901–1982) to the isolation of a bacillus (designated as *Bacillus palustris*) that produced an enzyme able to depolymerize the capsular polysaccharide of pneumococcus type 3 [3,4]. Potential drawbacks to the therapeutic use of this enzyme were the need for a specific enzyme for each capsular polysaccharide and the fact that, although effective for rendering the bacteria susceptible to phagocytosis, the enzyme could not be used to treat lobar infection.

Austrian concluded that treatment of pneumococcal infections has followed two, somewhat parallel courses: immunotherapy and chemotherapy [2]. The treatment of type 1 pneumococcal pneumonia with type-specific equine antiserum was initiated at the Rockefeller Institute in 1913 [3,4,6], where an approximately 50% reduction in mortality was shown in a mouse model of infection. Simultaneously, in 1911, J. Morgenroth and R. Levy reported the protective effect in mice of ethylhydrocupreine (optochin), a derivative of quinine [50]. Assays in vitro soon revealed bacterial resistance to optochin; resistance also occurred in humans, in whom the drug was briefly used as chemotherapeutic agent . Later, in the 1930s, sulfapyridine, a sulfonamide, proved to be moderately successful for treating pneumococcal pneumonia [45]. Capsular serotyping was largely abandoned with the introduction of penicillin treatment for pneumococcus and many other bacterial pathogens. In the case of pneumococcus, the overall fatality rate was reduced to 5–8% [2].

In the early 1920s, Frederick Griffith (1877–1941) was interested in defining the conditions under which unencapsulated variants of pneumococcus type 2 would regain capsulation and virulence in vivo. Using animal models, he observed that the simultaneous inoculation of mice with live avirulent bacteria (rough phenotype) and dead smooth virulent strains resulted in the isolation from dead mice of the smooth phenotype. The substance responsible for this unexpected phenotyp-

ic change was called "the transforming principle" [26]. Avery and his team took advantage of the natural transformation displayed by pneumococcus, namely, the capacity to incorporate some component from crude extracts of the smooth variant into the rough strain, to recreate and extend in vitro the observations made by Griffith (Fig. 1). In those experiments, previously unencapsulated strains acquired a capsule, becoming, in McCarty's words, "sugar-coated bacteria."

Further studies led to the successful genetic transformation of *S. pneumoniae* in vitro. The work of Avery's group was accelerated when he was joined by McCarty at his laboratory. McCarty's special skill as a biochemist to prepare highly purified DNA by using several pivotal enzymes, i.e., DNase and RNase, was fundamental to concluding that genes were made exclusively of DNA. In other words, DNA was the "transforming principle", as reported in the fundamental paper published in 1944 in *The Journal of Experimental Medicine*. No discovery arising from the study of bacteria has had a greater impact on biology than the finding that genes consist of DNA [5].

Regrettably, the discovery by Avery, McLeod, and McCarty was initially received with skepticism by other researchers and, unfortunately, was not quoted, 9 years later, in the now-famous paper by Watson and Crick describing the double-helix model of DNA [68]. During the celebrations commemorating the 50th anniversary of the proposal of the DNA double helix, Watson finally honored the fundamental importance of the discovery by saying: "And the fact that Avery, McLeod, and McCarty were not awarded the Nobel Prize is an oversight that, this day, still puzzles" [47]. This admission should be shouted from the rooftops!

From 1956 to the current state of the art. By the early 1950s, the wide-spread use of penicillin, the discovery of drugs to combat tuberculosis and, later, the development and generalized use of a vaccine against polio had remarkable effects on the attitudes of scientists and clinicians towards infections. Stanley Falkow well illustrates this situation: he recalled that, in the 1960s, several influential scientists suggested to him that studying microorganisms was a waste of time. One of these mentors, a Nobel laureate, even went one step further by asking: "Who cares anymore [about bacteria]?" [60]. Perhaps this complacency about microorganisms reached its highest level when the US Surgeon General stated that "the war against infectious diseases has been won". Instead, as stated in a recent review, microbiology "is now at the top of the life science agenda" [17].

These attitudes began to change in the early 1980s, when products encoded by bacterial genes and plasmids were found to interfere with the available drugs. At the time, research on pneumococcus was limited to the few basic subjects that, for more than 20 years, had attracted only a small number of scientists. From the 1970s until the mid-1980s, most research on *S. pneumoniae* focused on the peculiarities of genetic transformation, including competence development (a specialized physiological state that allows the incorporation of exogenous DNA), and recombination between recipient and exogenous DNA. Since then, a variety of experimental approaches, such as tracing the fate of isotopically labeled DNA, DNA cloning and sequencing, as well as identification of the surface receptor for competence factor and donor DNA, have facilitated an understanding of the fascinating phenomena that lead to transformation in pneumococcus
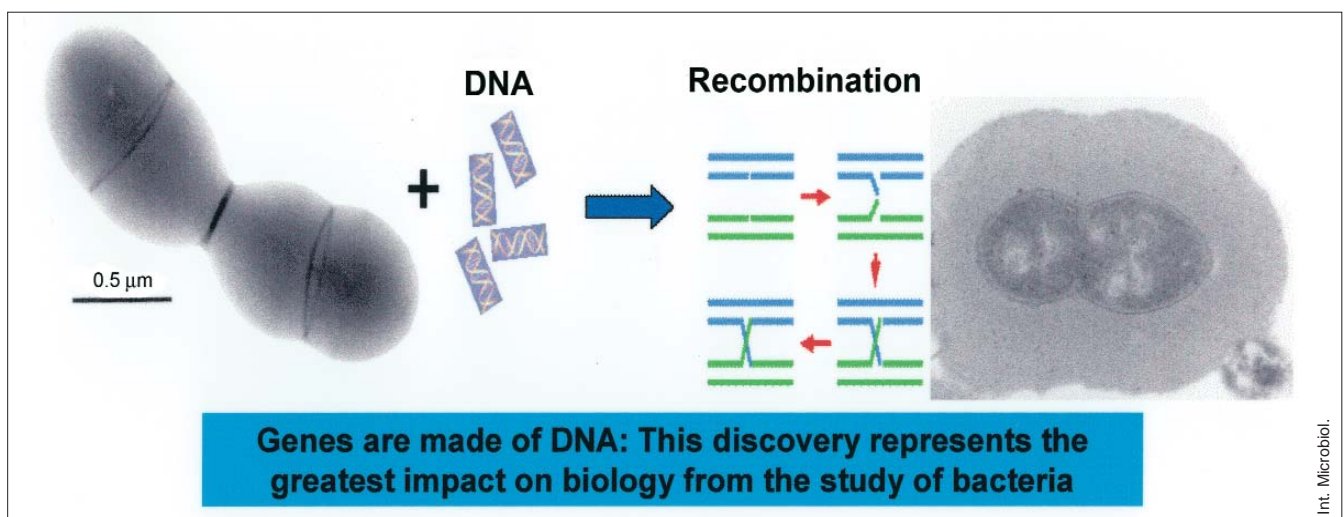

Fig. 1. Avery's historic genetic transformation experiment. An unencapsulated pneumococcal mutant (left) was incubated with purified DNA prepared from a fully encapsulated type-3 strain. After incubation and recombination, capsulated type-3 transformants were isolated (right).

[35,64]. The quorum-sensing signal responsible for competence induction is a heptadecapeptide, called CSP (competence-stimulating peptide), which derives from a precursor (*comC*) following cleavage and transport into the medium by an ATP-binding cassette transporter, ComAB. This peptide turned out to be CSP-1, since another peptide with similar function (CSP-2) was identified later on [29]. More recently, seven early and 14 late genes involved in genetic transformation have been identified, the former including genes encoding a two-component regulatory system (ComDE), a histidine kinase, which is also the CSP receptor, and a cognate regulator. CSP stimuli have also been linked with biofilm formation in *Streptococcus* species, mainly those forming dental plaques [63]. It has been suggested that DNA release [52] and bacterial agglutination properties together with competence development in *S. pneumoniae* [30] promote biofilm formation, thus favoring pneumococcal colonization. In fact, it was reported that DNA is one of the structural components of the extracellular matrix of biofilms and is required at early stages in the process of biofilm formation, as already reported for other bacteria such as *Pseudomonas aeruginosa* [69].

Penicillin resistance in pneumococcus is based on a complex mutational pathway that involves multiple alterations in several penicillin target proteins, the penicillin-binding proteins (PBPs). PBPs are responsible for the last enzymatic steps leading to formation of the cell wall [8]. In laboratory mutants, non-PBP genes also contribute to resistance whereas interspecies gene transfer of PBP variants between commensal streptococci and the pathogen *S. pneumoniae* appears to be responsible for the emergence of resistant clones via the formation of so-called mosaic genes. Pneumococcus strains that are resistant to many other antibiotics, including quinolones, have also been described [28].

These findings, together with the persisting morbidity and mortality associated with pneumococcal infections, led to the reintroduction of a vaccine program that used a polyvalent pneumococcal polysaccharide vaccine first tested in the 1960s. Based on the aggregate efficacy of a tetradecavalent formulation that included the 14 capsular types accounting for most of the infections in 1977, it was possible to prevent 78.5% of the infections originating from the types included in the vaccine. In 1983, the formulation of the vaccine was expanded to 23 capsular polysaccharides, the most complex vaccine ever administered to humans [2]. A case-controlled study comprising immunocompetent adults older than age 40 years showed an aggregate protection of about 61% with a clear decline in protection among those 65 years and older. It has also been well-documented that polysaccharide vaccines are suitable antigens for adults but they are not immunogenic in infants and young children. In the last few years, pneumo-

coccal polysaccharide-protein conjugates, analogous to that previously developed to combat infections caused by *Hemophilus influenzae* type b, have been produced. These vaccines have proven to be effective in preventing systemic infection with the capsular types included in the 7-valent preparation already licensed. However, field trials in humans are still needed to evaluate the prophylactic potential of these vaccines.

Contribution of our laboratory to knowledge of the molecular biology of the capsule, lytic enzymes, and bacteriophages of pneumococcus (1974-2006)

In 1973, I joined the group of Alexander Tomasz at The Rockefeller University and started to work with pneumococcus. For more than 30 years, my group has focused on three main aspects of this human pathogen: the lytic enzymes of the system, the characteristics of the pneumococcal bacteriophages, and the molecular analysis of its polysaccharide capsule (for a recent review, see [43]).

Lytic enzymes. Gram-positive bacteria are surrounded by several peptidoglycan layers (the glycan chain made of repetitive units of *N*-acetylglucosamine and *N*-acetylmuramic acid interlinked by peptide bonds), which give them their special shape. To allow the cell to expand, this rigid sacculum must continuously adapt. This cellular restructuring takes place through the action of murein hydrolases, which are endogenous enzymes capable of degrading peptidoglycan by cleaving covalent bonds of the cell wall. Cell-wall hydrolases (CWH) (or lytic enzymes) have been found in all eubacteria studied so far, and are thought to play major roles in the biology of bacteria, including cellular expansion, division, and separation of daughter cells. These enzymes are also crucial in microbial chemotherapy in that they are responsible for the irreversible effects caused by β-lactam antibiotics.

Pneumococcal and phage lysins hydrolyze specific bonds in the peptidoglycan network: the *N*-acetylmuramoyl-L-alanine amide bond between the glycan strand and the cross-linking peptide (NAM)-amidases; [EC 3.5.1.28], or the 1,4-β-linkage between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues of the glycan chain (lysozymes; EC 3.2.1.17) [24,43] (Fig. 2A). Glucosaminidases, transglycosylases, endopeptidases, and phosphorylcholins (PC) esterases encoded by pneumococcal phages have not been described, although *S. pneumoniae* synthesizes a glucosaminidase (LytB) and a PC esterase (Pce). Lytic transglycosylases differ from

Fig. 2. The cell wall of *Streptococcus pneumoniae* and the pneumococcal and phage cell-wall hydrolases. **(A)** Structure of the *S. pneumoniae* peptidoglycan. The different lytic enzymes are shown in parentheses. Cpl-1 and Cpl-7 are muramidases (lysozymes; EC 3.2.1.17) from phages Cp-1 and Cp-7, respectively. The (NAM)-amidases Pal, Hbl, Ejl, Mml, and LytA$_{VO1}$ are encoded, respectively, by phages Dp-1, HB-3, EJ-1, MM1, and VO1. Endopeptidases cleaving bonds other than those shown in the figure are also possible. TA, teichoic acid. Solid circles represent PC residues. **(B)** Six different molecular architectures of the lytic enzymes of the pneumococcal system. Sequence similarities among lysins are indicated by equal shading. Each protein is identified by its name, number of amino acid residues (aa), and accession number. Solid, gray, striped, and cross-hatched bars represent, respectively, lysozyme (Glyco_hydro_25 family; PF01183), amidase_2 (PF01510), amidase_5 (PF05382), and CHAP (PF05257) domains from the Pfam database. The substrate-binding domains, either a choline-binding domain (CBD) (PF01473) (open boxes) or not (PF08230) (stippled boxes), are also shown. Cpl-7 is a choline-independent lysozyme. Reprinted from *FEBS Lett.* [39] with permission of the publisher.
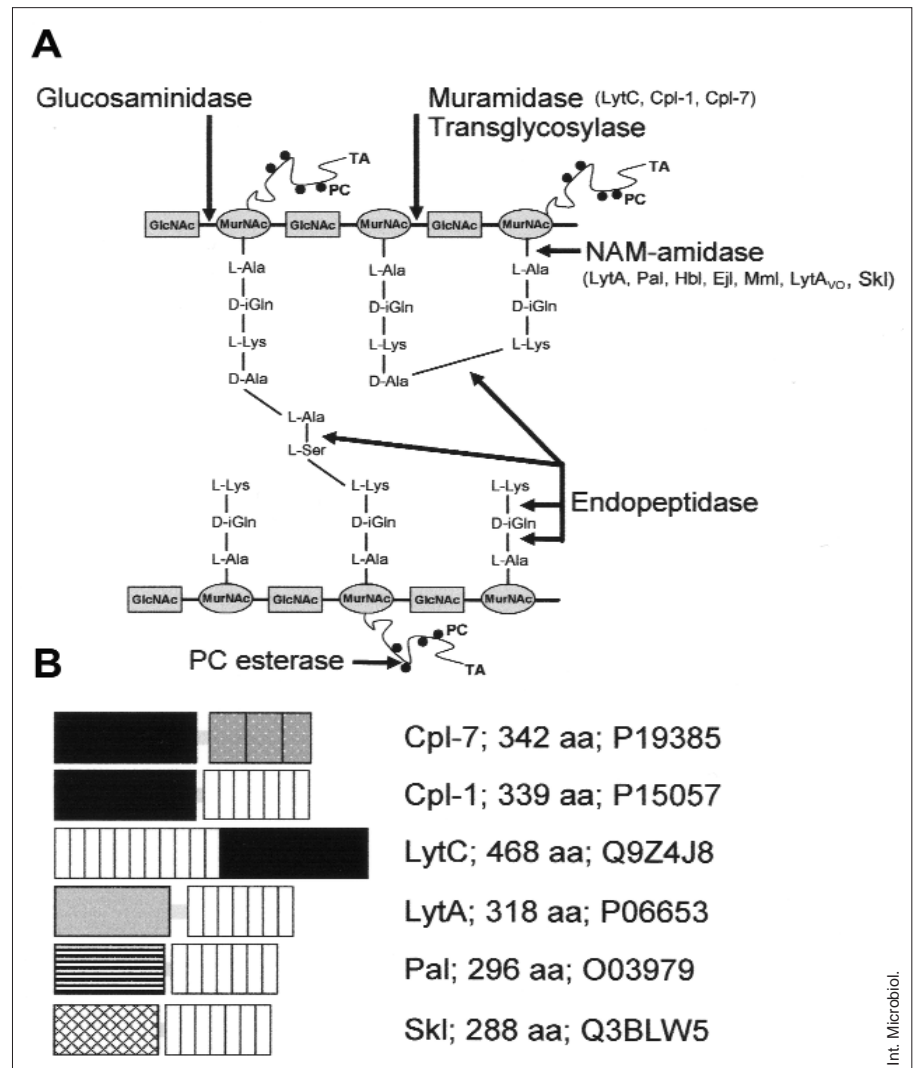
lysozymes by catalyzing intramolecular transglycosylation of the glycosyl bond onto the C6 hydroxyl group of the muramic acid residue, thus forming a 1,6-anhydromuramic acid derivative. All these enzymes have in common an absolute dependence for activity on the presence of choline in cell-wall teichoic acid, the so-called choline-binding proteins (CBPs). These proteins have a modular organization and one of the modules consists of motifs that recognize choline (CBD) (see below). We have taken advantage of the choline analogue diethylaminoethanol (DEAE) to easily isolate these CBPs on DEAE-cellulose columns. In this technique, developed some time ago by our team, CBPs are selectively retained [43].

In addition to *S. pneumoniae* and its phages, four prophages from *Streptococcus mitis* also contain genes encoding CBPs with lytic activity, namely, the NAM-amidases from EJ-1 (Ejl), SM1 (gp56), φB6 (LytA$_{B6}$), and φHER (LytA$_{HER}$) [24]. Gp56 is very similar (72% identical; 85% similar) to the Dp-1 NAM-amidase (Pal) whereas Ejl, LytA$_{B6}$, and LytA$_{HER}$ are more than 80% identical to the NAM-amidases LytA, Hbl, MMl, and LytA$_{VO1}$ encoded by *S. pneumoniae* and its temperate phages HB-3, MM1, and VO1, respectively [24]. Although many CBPs with lytic activity have been reported, note that only six different molecular designs have been reported so far (Fig. 2B). Four CWHs (LytA, LytB, LytC, and Pce) have been dissected in detail in pneumococcus. LytA is the major lytic enzyme and plays a critical role in the biology of pneumococcus, as discussed above. Crystal structure analyses revealed that the pneumococcal C-terminal LytA domain (C-LytA) displays a solenoid structure made up exclusively of β-hairpins that pile up to form a left-handed superhelix [18,19]. Every hairpin corresponds to the motif that we had defined by analyzing the protein's primary structure. For the enzyme to achieve its structure, each choline molecule must locate in the hydrophobic interphase formed by the con-

secutive hairpins. In addition, the active version of the enzyme requires the formation of a dimer with a peculiar boomerang structure. LytC and LytB have similar structural organizations, in which, unlike LytA, the region for choline recognition is located at the N-terminal domain, consisting of 11 and 18 repeated motifs, respectively; in contrast to LytA, they also have signal peptides. Inactivation of the gene *lytB* leads to the formation of long cell chains (Fig. 2A) [12].

The first pneumococcal lysozyme described in pneumococcus was LytC. Biologically, it works as an autolysin when cultures are incubated at 30ºC. As the carrier state of pneumococcus is located in the upper respiratory tract, usually a well-ventilated region of the body (ca. 34ºC), LytC might play an important role in the natural transformation processes occurring at this location. Likewise, we have observed that cells lacking this enzyme tend to form clusters.

We have purified LytB, which has been identified as a glucosaminidase. Purified LytB added to cultures of pneumococcus *lytB* mutants, which form characteristic long chains, promoted dispersion of the bacteria into diplococci or short chains, the typical morphology of wild-type pneumococcal strains [12]. In addition, the preparation of chimeric enzymes by means of a translational fusion between *gfp,* the gene coding for the green fluorescent protein (GFP), and *lytB* showed that LytB accumulates in the cell poles, where it might very selectively lyse the cell wall. Variations in the composition of choline motifs might account for the selective recognition of LytB; for example, there may be specific receptors for this enzyme at the polar region of the cell surface, where peptidoglycan hydrolysis would take place. This could explain why LytB, unlike LytA and LytC, does not behave as an autolytic enzyme. Since cellular dispersion might be a major factor in virulence, the lack of LytB might impede pneumococcal dissemination during the infective process.

In vitro experiments carried out with purified Pce confirmed that this enzyme is a teichoic acid (TA) phosphorylcholine esterase able to remove a maximum of only 20% of the phosphorylcholine residues from cell-wall TA, in agreement with earlier results [11]. As it is also bound to the envelope, Pce should play its role only after being secreted through the membrane, although it is currently difficult to assign a defined function to this enzyme. It has been suggested that the 20% fraction of residues removable by the enzyme exists either in an anatomically unique position in the cell wall or represents terminal residues in the TA chains [11,66]. This esterase activity might regulate the availability of choline residues required for activity (attachment) of its own and/or of other CBPs. Recently, a novel choline-binding NAM-amidase (Skl) of *S. mitis* SK137 containing a CHAP (cysteine, histidine-dependent amidohydrolase peptidase) motif has been characterized [39].

Pneumococcal bacteriophages. Bacteriophages are the most abundant entities in the biosphere (about 10^{31}), and detailed studies carried out in different bacterial species have shown that phages can be major vehicles for the transmission of virulence genes within bacterial populations.

It was not until 1975 that a phage was first isolated in pneumococcus by two independent groups, phage Dp-1 by C. Ronda and M. McDonnell at the laboratory of A. Tomasz, and phage ω1 by G. Tiraby at the laboratory of M. Fox. Six years later, the Cp (Complutense phage) family was isolated in our laboratory (for a recent review see [24]). Since then, we have analyzed a series of lytic and temperate phages. Our team also showed that the presence of choline in cell-wall receptors was essential for the adherence of some phages, including Dp-1. In fact, if choline was replaced by ethanolamine, pneumococcal phages could not adsorb and the cell was resistant to lysis by bacterial or phage-encoded lysins. More recently, the first complete genomes of two lytic (Dp-1 and EJ-1) and two temperate (MM1 and EJ-1) phages have been sequenced. We were therefore able to determine the functional organization of the genome. These clusters contained several genes of great interest that are the focus of our current research. This is the case for a gene found in the genome of Dp-1 (*orf55,* coding for the antireceptor) that codes for a protein containing motifs similar to those found in CBPs for the recognition of choline in the cell wall. This observation explains the requirement of choline by Dp-1 phage receptors for adsorption [24]. Up to now, we have also described the structural organization of four CBPs identified as CWHs. These proteins are responsible for the specific recognition of choline units and are the lytic phage proteins that liberate progeny from infected *S. pneumoniae* (Fig. 3).

The four bacterial lytic enzymes described so far have great intrinsic flexibility, which enables them to shift recognition units from the C-terminal region to the N-terminal one. This ability fulfils the exchanging, functional properties that R.F. Doolittle attributes to a well-defined domain [16]. We also observed that the murein hydrolase of the Cp-7 phage, a phage very similar to Cp-1, is an exception to the choline dependence of phage lytic enzymes, since it is able to degrade the pneumococcal wall in the absence of this aminoalcohol. These features are reflected in the enzyme's primary structure, because the peculiar motifs for choline recognition have been replaced by three, identical 48-amino acid long motifs.

Direct experimental evidence for our hypothesis regarding the modular organization of enzymes of the pneumococcus system was obtained by constructing chimeric functional phagebacterial lytic enzymes [13,23]. These showed new functional characteristics that were the result of exchanges with the
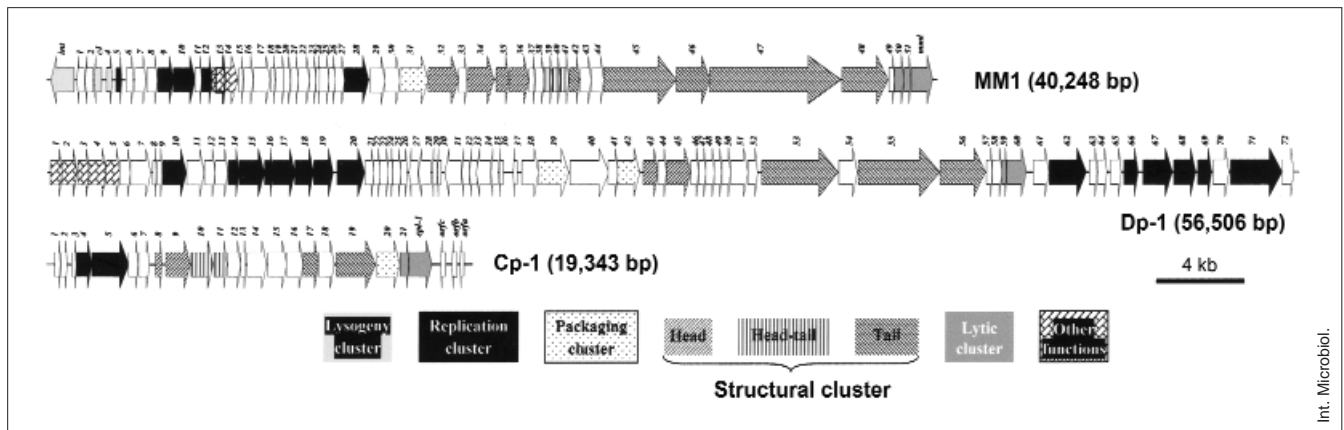
Fig. 3. The genomes of three pneumococcal bacteriophages, MM1, Dp-1, and Cp-1. Genes are drawn as arrows that indicate the direction of transcription. White arrows correspond to ORFs that do not have any significant similarity with those included in databases. For MM1, the ends of the genome correspond to those of the prophage. The putative functions of the gene products correspond to the different types of shading, as indicated at the bottom. Reprinted from [24] with permission of the publisher.

parental enzymes. We also produced intergeneric chimeric enzymes with *Clostridium acetobutilicum* [10]. The results allowed us to postulate that this kind of exchange could provide the pneumococcal system with high-plasticity mechanisms to yield new enzymatic combinations in nature, by means of simple genetic recombinations that evolution would refine, and from which pneumococcus would benefit evolutionarily. The isolation, cloning, and purification of Dp-1 phage lytic enzyme (Pal) offers an example that supports this working hypothesis. This enzyme, which we have characterized as an NAM-amidase, has an N-terminal region very similar to that of a lactococcus phage amidase, whereas the C-terminal domain is highly similar to those able to recognize choline-containing substrates. Thus, the formation of a natural intergeneric chimera allowed a primordial enzyme, possibly without a recognition unit, to obtain such a unit in order to improve its catalytic efficiency.

Since 1927, it has been known that during the lysogenic state of temperate phages certain genes that code for toxins are expressed. These toxins are usually the main cause of bacterial virulence, as is the case, for example, in scarlet fever. Initial results reported that filtered supernatants of toxicogenic streptococcal cultures acquired the ability to produce scarlatinal toxin, were in fact describing transduction, the transfer of genetic material to a bacterial cell via phage infection, even though investigators lacked an explanation for this phenomenon [67]. Subsequently, their hypothesis, that bacteria acquire virulence properties from phages, has been widely accepted. In fact, it has been shown that many virulence genes are transferred among bacteria by phages (via transduction) and other mobile genetic elements, such as plasmids (via conjugation), as well as by incorporation of the phage genome into the bacterial chromosome. These types of observations were later extended to cholera and diphtheria;

nowadays the list is abundant [67]. Most probably, this is also the case in *S. pneumoniae*. In fact, 70% of the pneumococcal genomes from clinical isolates contain prophages (or remnants of them). How these phages contribute to pneumococcal virulence is under investigation in our laboratory. We speculate that the mechanisms of virulence in pneumococcus follow patterns others than those described thus far.

Since their discovery by d'Hérelle and Twort some 90 years ago, phages have been used as antibacterial therapy in Eastern Europe [62]. A brilliant experimental variant to phage therapy was developed recently by Fischetti et al. [42], in which phage products, i.e., lytic enzymes, were used. In the case of pneumococcus, these assays included the Pal amidase and the Cpl-1 lysozyme. The lytic enzymes used in this experimental approach were *enzybiotics* designed to achieve cure at the carrier stage in a murine model of pharynx infection by the administration of instillations of these purified enzymes. More recently, this experimental approach was extended to *Bacillus anthracis*, with the aim of fighting anthrax, a pathogen that has raised great concern due to the fear of bioterrorism, and to *Streptococcus pyogenes* [44].

In the case of *S. pneumoniae*, collaboration between my team and that from the Instituto de Salud Carlos III, in Madrid, resulted in a murine septicemia model, in which a single dose of Cpl-1 lysozyme or Pal amidase was shown to protect experimental animals from fatal infection by a virulent, clinical pneumococcus. So far, no adverse reactions to treatment with these murein hydrolases have been observed [32]. These results are encouraging as a promising new therapeutic approach to lessen the alarming antibiotic resistances that have evolved in numerous bacterial species, especially pneumococcus, due to the genetic plasticity of bacteria and to the misuse of these drugs.

Capsular polysaccharide. Pneumococcal capsules are polysaccharides excreted outside the cell. They are usually composed of repeating units of simple sugars that remain attached, probably in a covalent form, to the outer surface of the bacterium. Capsules are usually associated with increased virulence as they may function as adhesins, recognition molecules, and/or by favoring the camouflage of the parasite against the host immune response. The capsule of polysaccharide that completely envelops *S. pneumoniae* acts as a protective layer that isolates the bacterial cell from the environment.

A remarkable combination of a series of genes at the capsular cluster results in at least 90 structurally and serologically distinct capsular polysaccharides (serotypes) that contribute to the clinical importance of *S. pneumoniae*. Although the biochemistry of some serotypes has been known for a long time, it was not until the early 1990s that the molecular bases of capsule formation in several bacteria species began to be understood. In 1993, my group reported the location and isolation of a gene coding for serotype-3 capsule [22]. Since the late 1950s, it had been known that genes coding for the pneumococcal capsule formed a cluster (Fig. 4). The localization and isolation of those genes (*cap/cps*) showed, as biochemists had suggested, that the greater the complexity of the capsule's composition, the more genes involved in its formation. This proposal was fully confirmed upon molecular analysis.

According to the most recent data, there are three different organizational models of the capsular gene cluster in *S. pneumoniae* (for a review, see [38]):

(i) The most common capsular gene cluster organization corresponds to that of types 1, 2, 4, 6B, 8, 9V, 14, 18C, 19F, 19A, 19B, 19C, 23F, and 33F. Moreover, sequencing of the genes encoding the already-known 90 pneumococcal serotypes [31] is currently underway at the Sanger Institute [http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS]. Most of the gene clusters share a similar organization. The *cap/cps* gene cluster is located between *dexB* and *aliA*, two genes that do not participate in capsular biosynthesis (Fig. 4). In all cases, a functional promoter is located immediately upstream of the gene cluster [1,40,53], and the first four open reading frames (ORFs) of the *cap/cps* operon are well-conserved among serotypes, although only the first ORF is virtually identical in all of the types analyzed so far. In spite of the sequence conservation of the two first ORFs among serotypes, these genes show enough polymorphism to allow the serotyping of *S. pneumoniae* isolates by PCR-based methods [34,36,48]. In this model of capsular clusters, the mechanisms of regulation and transport of capsular polysaccharide (CP) have been recently studied. Production of most CPs is achieved through the formation of a lipid-linked repeat unit that is synthesized on the intracellular face of the membrane,

exported to the surface, and polymerized. It has been reported that deletion of either *cps2A*, *2B*, *2C*, or *2D* genes in the serotype 2 gene cluster does not affect the transfer of CP to the cell wall [7]. Interestingly, the correlation between tyrosine phosphorylation of CpsD and CP production is a matter of current debate. It was first reported that CpsD acts as a negative regulator of capsule biosynthesis [51].

(ii) Type 3 is an exceptional gene cluster in that the four initial ORFs of the capsular operon are not involved in CP biosynthesis and are not expressed [1] (Fig. 4). A functional promoter is located immediately upstream of the first gene of the operon (*cap3A*). In accordance with the simple chemical structure of the type 3 repeating unit [cellobiuronic acid units connected in a $\beta(1\rightarrow3)$ linkage], only three complete genes were found in the capsular operon (Fig. 4). Moreover, the third gene (*cap3C*; also referred to as *cps3U*), is not required for CP biosynthesis since the biochemical function of its product (a UDP-Glc pyrophosphorylase) is compensated by that of the *galU* gene, located far away in the *S. pneumoniae* chromosome [49]. The GalU enzyme has been shown to be essential for CP synthesis and is required for the interconversion of UDP-Glc and UDP-galactose by way of the Leloir pathway [20]. Prokaryotic UDP-Glc pyrophosphorylases are well-conserved and, although UDP-Glc pyrophosphorylases are also present in eukaryotes, these enzymes are completely unrelated to their prokaryotic counterparts ([49] and references therein).

(iii) The most peculiar case among pneumococcal capsular genes is provided by type 37 isolates. These strains are genetically binary; that is, they contain a *cap37* locus virtually identical to that of type 33F strains [40,41] but several mutations have inactivated some of the ORFs and, consequently, this locus is actually silent. It was also demonstrated that type 37 capsulation is due to the presence of a single copy of a gene (*tts*) located far apart from the *cap* cluster (Fig. 4). The Tts synthase contains several motifs known to be characteristic of cellulose synthases and other glucosyltransferases [37].

In a bacterium with natural genetic transformation, the modular structure of the pneumococcal capsule facilitates the exchange of specific genes between serotypes by means of recombination between flanking homologous regions. This exchange, evidenced by Coffey et al. [9], is of great clinical importance, particularly when it occurs between antibiotic-resistant strains. In fact, this implies an epidemiological challenge in controlling the universal expansion of some pneumococcal strains. In addition, the fact that changes in capsular type by recombination might be relatively frequent among pneumococci will impact the long-term efficacy of conjugate pneumococcal vaccines, which will protect only against a
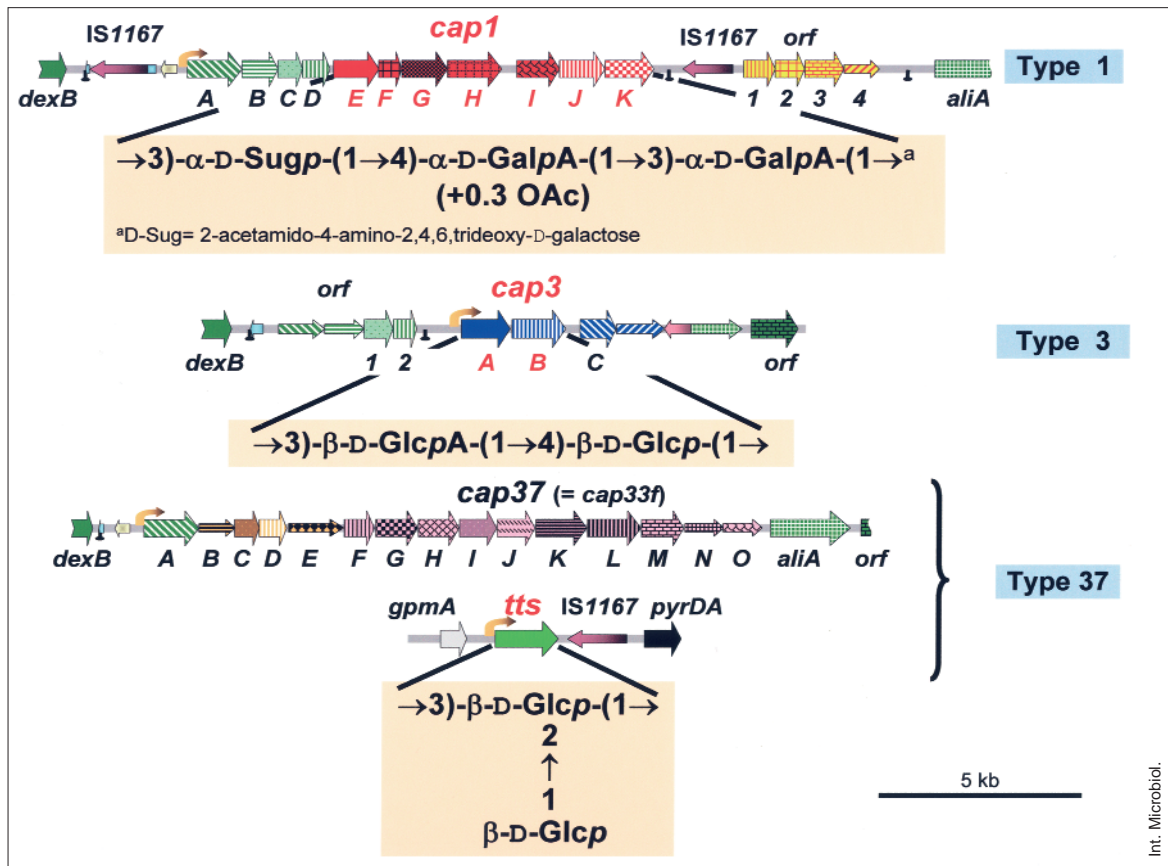
Fig. 4. Genetic organization of the *S. pneumoniae* region containing the *cap/cps* gene cluster of three representative capsular types. Large and thin arrows represent complete or interrupted ORFs, respectively. Regions showing more than 90% identical nucleotides are represented by identical color and shading. The 'inverted matchsticks' represent putative transcription terminators. Bent arrows show the location of functional promoters. The composition of the monomeric unit of the capsular polysaccharide (CP) encoded by the three represented serotypes is also shown. Black lines surround the genes of the capsular cluster coding for these polysaccharides. The inactive *cap37* (= *cap33f*) cluster present in type 37 pneumococci is also shown. Reprinted from *FEMS Microbiol. Rev.* with permission of the publisher. [López, García (2004) FEMS *Microbiol. Rev.* 28:553-580]

limited number of serotypes [65]. Besides the general mechanism that controls capsule formation, transposition-like events may contribute to capsular diversity in *S. pneumoniae*, as evidenced by the fact that all the capsular gene clusters of *S. pneumoniae* are flanked by insertion sequence elements.

Sequencing of the *galU* locus has provided insight into a gene implicated in the synthesis of all known pneumococcal serotypes. GalUprotein is thus an ideal metabolic target for future clinical approaches that would involve blocking capsule formation by *S. pneumoniae*. It is indeed an interesting candidate for the design of a conjugate vaccine.

Future prospects

The global importance of *S. pneumoniae* as the cause of illness, adverse sequelae, and death, as well as the emergence of drug resistance that is making these infections more difficult to treat, justify the current interest of many research teams in this major human pathogen. Gene flow in natural populations needs to be further examined to understand the continuous evolution of multiple antibiotic resistances. The role of vectors, including conjugative transposons and bacteriophage, needs to be addressed in a clinical setting. The potential emergence of new pathogens from related oral microbiota needs to be examined through better characterization of these organisms as donors of genetic material to the pneumococcal gene pool and as organisms that could emerge within the pneumococcal nasopharyngeal habitat. Mechanisms and environmental stresses promoting genetic transformation and mutational events among pneumococci and between pneumococci and the related oral microbiota also deserve further study.

There is also the problem of the poor efficacy of vaccines

due to the well-documented genetic plasticity of *S. pneumoniae*. In addition, eradication of pneumococcus through the generalized use of antibiotics looks to be unrealistic, again because of the bacterium's genetic plasticity, which results in the rapid appearance and spreading of antibiotic-resistant isolates and antibiotic resistance "determinants". Moreover, because *S. pneumoniae* is a human commensal, it may not be sound to disturb the normal flora, with unpredictable long-term consequences. Finally, it is widely recognized that biofilm formation is the natural form of growth for most microbial species in their natural habitats [27]. A biofilm is a highly structured sessile microbial community characterized by bacterial cells attached to a surface or interface and embedded in a matrix of extracellular polymeric substances [15]. To ascertain whether *S. pneumoniae* benefits from forming an interactive community and whether this sessile mode of growth is the result of random accretion of bacterial cells or of a community of bacteria cooperating to form a well-defined structure, we studied the structural peculiarities of these biological entities by using laboratory conditions that favor biofilm formation. Our results provided evidence that pneumococci form biofilms on abiotic surfaces. Furthermore, we studied conditions that lead to defective biofilm development by using genetic approaches focused on the earliest stage of biofilm accretion, that is, the surface attachment of *S. pneumoniae*, before an ordered three-dimensional structure is constructed. Our analyses revealed the role of certain gene products in primary attachment during biofilm formation and suggested that DNA and several proteins contribute to the formation of extracellular matrix in this system. These experimental data are important steps in furthering our understanding of the putative role of biofilms in some stages of pneumococcal infection and their possible contribution to increasing antibiotic resistance in the clinical setting.

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Pneumococcus: la bacteria recubierta de azúcar

Resumen. Hasta el empleo generalizado de los antibióticos, el estudio de *Streptococcus pneumoniae* (el neumococo) fue un tema central en medicina durante muchas décadas. Muchas contribuciones fundamentales de la historia de la microbiología se deben a esta bacteria: la reacción capsular de la precipitina, su destacado papel en el desarrollo de la inmunología mediante la identificación de polisacáridos como antígenos, y, principalmente, la demostración, por transformación genética, de que los genes están compuestos de DNA, que supuso el mayor impacto en biología a partir del estudio de las bacterias. Actualmente, el neumococo es el agente etiológico más frecuente en procesos de infección aguda del oído medio, sinusitis y neumonía que requieren hospitalización en adultos. Además, la meningitis es una de las principales causas de muerte en los niños de países en vías de desarrollo. Esta revisión trata las contribuciones que han llevado a un elevado nivel de conocimiento sobre el neumococo y describe algunas contribuciones de nuestro grupo al conocimiento de la base molecular de los tres factores principales de la virulencia, es decir, las enzimas líticas, los fagos y los genes que codifican los polisacáridos capsulares. [**Int Microbiol** 2006; 9(3):179-190]

Palabras clave: *Streptococcus pneumoniae* · polisacárido capsular · hidrolasas de pared celular · bacteriófagos · factores de virulencia

Pneumococcus: a bactéria recoberta por açúcar

Resumo. Até o emprego generalizado dos antibióticos, o estudo de *Streptococcus pneumoniae* (o neumococo) foi um tema central em medicina durante muitas décadas. São numerosas as contribuições fundamentais na história da microbiologia devidas a esta bactéria: a reação capsular da precipitina, seu papel no desenvolvimento da imunologia através da identificação de polissacarídeos como antígenos, e, principalmente, a demonstração, pe transformação genética, que os genes são compostos por DNA, o que representou o maior impacto em biologia a partir do estudo das bactérias. Atualmente, o neumococo é o agente etiológico mais comum em processos de otite aguda, sinusites e pneumonia que requerem hospitalização em adultos. No entanto, é a meningite a causa principal de morte em países desenvolvidos e em crianças em países em vias de desenvolvimento. Nesta revisão, propomos também uma análise das contribuições que levaram ao elevado nível de conhecimento sobre o neumococo, assim como um breve relatório sobre as contribuições de nosso grupo à base molecular dos três principais fatores de virulência isto é, os enzimas líticos, os fagos os genes que codificam os polissacarídeos capsulares. [**Int Microbiol** 2006; 9(3):179-190]

Palavras chave: *Streptococcus pneumoniae* · polissacarídeos capsular · hidrolases de parede cellulare · bacteriófago · fatores de virulência