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LYSIS STRATEGY OF *STREPTOCOCCUS PNEUMONIAE* BACTERIOPHAGES: MECHANISMS AND HOST IMPLICATIONS

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DOUTORAMENTO EM CIÊNCIAS E TECNOLOGIAS DA SAÚDE

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And last, I am eternally grateful to Ricardo to light up my day!

Maria João Frias

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SUMMARY

Keywords: *Streptococcus pneumoniae*, phages, lysis, autolysin

Bacteriophages (phages), the most abundant entities in the biosphere, play a central role in the shaping of natural populations of bacteria. Phages have also been the focus of several studies due to their potential as tools for therapeutic purposes. Notably, detailed analysis carried out in different bacterial species established that phages have a prominent influence in virulence. The abundance of lysogenic phages in *Streptococcus pneumoniae* isolates associated with infection was suggested some years ago, and recently, it has been proposed that lysogens account for as much as 76% of the samples analyzed. However, the role of pneumococcal prophages in the pathogenic potential of its host remains so far unknown.

Bacterial lysis promoted by the major autolysin LytA has been implicated in the capacity of pneumococcus to cause infection, essentially due to the release of proinflammatory cell wall compounds and intracellular virulence factors. Even if no phage-encoded virulence factors were ever found, prophage-mediated host lysis by itself may contribute significantly to pneumococcal pathogenesis. Therefore, investigating the phage lysis system is clearly important in furthering our understanding of this effect. This work explores the exact mechanism underlying the lysis strategy of *S. pneumoniae* phages to release their progeny and also the implications of lysogeny, particularly due to induced cell lysis, in the host ability to form biofilms, a bacterial lifestyle associated with pneumococcal human infections.

Pneumococcal phages lyse their bacterial hosts, and consequently release the newly formed phage particles, at the end of the vegetative cycle through the combined action of holins that form lesions in the cytoplasmic membrane and lysins that degrade the bacterial peptidoglycan. The powerful lytic activity of the *S. pneumoniae* autolysin raised the possibility that it could play an important role in this process.

By deleting the bacterial and phage lysins in both lysogenic and lysogenized strains, the contribution of LytA to phage release was evaluated based on bacterial culture lysis monitoring and phage plaque assays. It was found that, independently of the host genetic background, the bacterial autolysin is activated during phage-mediated lysis. Flow cytometry assessment of the membrane integrity after phage induction revealed that LytA triggering results from holin-induced membrane disruption, similarly to the activation of the phage lysin. These results provide evidence that the energy status of the membrane may be involved in autolysin regulation at the cell surface.

We were able to demonstrate that, in the absence of the phage lytic enzyme, LytA by itself mediates extensive bacterial lysis, accompanied by the release of a large amount of fully functional phages capable of completing their life cycle since phage plaques were clearly detected. The overwhelming majority of phages of other bacterial species are absolutely incapable of bacterial lysis, trapping the phage progeny within the host cell, when the genes encoding lysins are deleted. Moreover, those rare mutants that bring about lysis depend only on phage-encoded factors. Nevertheless, exclusive dependence on the autolysin delayed the lysis timing and reduced the lysis extent. Accordingly, phage plaques were detected later than those in the presence of both host and phage lysins and a significant decrease on the number of virions released was observed. Therefore, lysis strictly dependent on LytA can lead to phage fitness impairment by retaining phage progeny longer within the host and reducing the amount of particles that actually escapes from entrapment. Nonetheless, under normal conditions, it was found that the concurrent activation of LytA with the phage lysin increases the total number of phages that exit the cell when the infective cycle is completed. Hence, pneumococcal phages use the ubiquitous host autolysin to accomplish an optimal phage exiting strategy and are unique among lysin-equipped phages in their dependence on bacterial lytic factors to achieve such task.

Although the function of holin and phage lysin is characterized, the interplay between them to achieve lysis in *S. pneumoniae* was never fully determined. It has been shown that pneumococcal phage lysins are structurally and functionally similar to LytA, thus, they may share the same cellular localization and control mechanisms. Our finding that holin-induced membrane lesions trigger the bacterial cell wall autolysin prompted a deeper study of the pneumococcal lysis strategy.

For this purpose, deletions of the holin and autolysin were performed in a lysogenic strain, in which the resident phage has a typical holin-lysin cassette. In the absence of these functions, western blot analysis and the effect of membrane permeabilizing and proton motive force (pmf)-dissipating agents on culture lysis allowed concluding that pneumococcal phage lysins accumulate with time across the lytic cycle and are continuously targeted to the cell wall. The phage lysin remains inactive associated with the choline residues within this structure. Therefore, the access of pneumococcal phage lysins to the bacterial surface is holin independent, hence they can be classified as exolysins. These findings are in contrast to what is observed in the large majority of holin-lysin phages where endolysins accumulate in the cytoplasm since they lack an intrinsic secretory signal sequence and consequently depend on holins to reach the peptidoglycan target. In addition, the involvement of the host Sec pathway in the phage lysin export was investigated. We assessed the cell wall localization of the phage

lysin by the same experimental procedures after culture treatment with the Sec inhibitor sodium azide. It was found that the phage lytic enzyme is possibly exported by the Sec system of pneumococci in spite of the striking absence of a signal sequence that could target it to the extracytoplasmic environment. This may constitute the first evidence, on phages encoding only holins and lysins in their lytic cassettes, of an exolysin without a secretion signal that is translocated through the membrane by the host Sec machinery. Furthermore, since the cell wall located autolysin also lacks obvious motifs or signals for an external localization, these results may provide clues for its transport mechanism.

Dependence exclusively on the pmf-dissipating agent for complete host lysis, together with the previous observation of holin's permeabilizing effect, showed that collapse of the cytoplasmic membrane electrochemical gradient achieved by the holins is the triggering signal to activate the phage lysin. In this study, it was further confirmed that activation of the externalized bacterial autolysin LytA, previously shown to contribute to phage progeny release, is also equally related to perturbations on the energized membrane. Thus, these results demonstrate that in *S. pneumoniae* phages, holin is not required for lysin export but is crucial to trigger the phage and bacterial lysins already residing in the cell wall by pmf dissipation upon formation of lesions on the membrane. In this regard, holins are the timing device that dictates when lysis takes place.

After the characterization of the phage lytic mechanism, the contribution of lysis mediated by lysogenic phages to pneumococcal biofilms was investigated. *S. pneumoniae* lysogens are associated with human infections and pneumococcal biofilms have been implicated both in colonization and infection. It was explored if prophage spontaneous induction and consequent bacterial lysis enhance pneumococcal biofilm development providing a source of extracellular DNA (eDNA), a major factor in the biofilm matrix.

Monitoring biofilm growth of lysogens and nonlysogenic bacteria by biomass quantification, viable cell counts and confocal laser scanning microscopy (CLSM), indicated that lysogenic bacteria are more prone to form biofilms, yielding structures with higher biomass and cell viability and showing denser biofilms in CLSM. Spontaneous phage induction was demonstrated to occur continuously as phages could be detected throughout biofilm formation through measurement of the total number of PFUs (plaque forming units) at specific time points. When comparing biofilm development between wild-type lysogens and those deleted in the phage lysin, bacterial autolysin LytA or both lysins, it was observed that phage-mediated lytic events influence positively the biofilm structure. These results established that prophage promotes biofilm development due to bacterial lysis upon spontaneous induction. In

agreement, lysis inside biofilms also occurs in other bacterial species and it might be related to increased biofilm fitness.

However, the effects created by the ablation of either the phage or bacterial lysins were overcome by the addition of external DNA. Additionally, in independent experiments, it was found that treatment with DNase I resulted in sparser and thinner biofilms while supplementation with DNA resulted in a thicker and more densely packed structure, confirming the important role of eDNA in pneumococcal biofilms. The quantification of eDNA released within these structures by real-time PCR also supported that lytic events promoted by phage are an important source of this matrix component, as biofilms of lytic strains contained more eDNA than those of nonlytic strains. Therefore, limited phage-mediated host lysis constitutes an important source of eDNA in *S. pneumoniae* biofilms favoring biofilm formation by lysogenic strains. Interestingly, massive phage induction leading to a high proportion of lysis was observed to disrupt severely biofilms of pneumococcal lysogens with a significant decrease in biofilm mass confirmed by CLSM visualization. These findings corroborate previous studies that show the potential use of lytic phages to destroy bacterial biofilms.

The presented results and conclusions are of great value not only to directly increase our knowledge on phage biology and their relationship with the host bacteria, but ultimately to uncover the role of lysogeny in pneumococcal virulence. In this context, massive prophage-induced lysis of the host could mimic the major bacterial autolysin by releasing factors known to contribute to the course of infection. On the other hand, lysis due to spontaneous levels of induction, characteristic of prophage carriage, may have an impact in pathogenesis by enhancing *S. pneumoniae* biofilm formation, which has been implicated in the processes of colonization and disease. A deeper understanding of the mechanisms underlying pneumococcal infection is of vital significance to manage this important human pathogen.

RESUMO

Palavras-chave: *Streptococcus pneumoniae*, fagos, lise, autolisina

Os bacteriófagos (fagos) são entidades extremamente abundantes na natureza que desempenham um papel central na modulação das populações bacterianas. Devido ao seu potencial como ferramentas para fins terapêutico, os fagos têm sido alvo de diversos estudos. A análise detalhada realizada em diferentes espécies bacterianas permitiu estabelecer que os fagos têm uma influência marcante na virulência. A abundância de estirpes lisogénicas de *Streptococcus pneumoniae* responsáveis por infecção foi sugerida há alguns anos e, recentemente, foi proposto que correspondem aproximadamente a 76%. No entanto, o papel dos profagos no potencial infeccioso dos pneumococos é ainda desconhecido.

A lise bacteriana promovida pela principal autolisina LytA foi implicada na capacidade de *S. pneumoniae* causar infecção sobretudo por promover a libertação de componentes da parede celular com actividade pro-inflamatória e factores de virulência intracelulares. Mesmo que, até à data, não tenham sido identificados factores de virulência nos genomas fágicos, a lise bacteriana mediada pelos profagos pode por si só contribuir significativamente para a patogenicidade do pneumococo. Desta forma, é importante estudar o sistema de lise dos fagos. O presente trabalho explora os mecanismos da estratégia de lise adoptada pelos fagos de *S. pneumoniae* para libertar a descendência fágica e as implicações da lisogenia, particularmente devido à indução de lise, na capacidade do hospedeiro formar biofilmes, uma forma de crescimento bacteriano associada com infecções pneumocócicas.

Os fagos de *S. pneumoniae* lisam as células hospedeiras, e consequentemente libertam as partículas fágicas recém-formadas, no final do ciclo lítico através da acção conjunta de holinas que formam lesões na membrana citoplasmática e lisinas que degradam o peptidoglicano da bactéria. No entanto, como a autolisina de *S. pneumoniae* é caracterizada por uma extensa actividade lítica, é possível que possa desempenhar um papel importante neste processo.

A contribuição de LytA na libertação dos fagos foi avaliada através da eliminação das actividades das lisinas fágica e bacteriana em estirpes lisogénicas e lisogenizadas, subsequente acompanhamento da lise das culturas bacterianas e realização de ensaios de placas fágicas. Foi determinado que, independentemente do contexto genético do hospedeiro, a autolisina bacteriana é activada durante a lise mediada pelo fago. A avaliação da integridade da membrana por citometria de fluxo após indução do fago revelou que a activação de LytA resulta, tal como a activação das lisinas fágicas, dos danos na membrana induzidos pelas holinas. Estes resultados sugerem que o estado energético da membrana está envolvido na regulação da autolisina na superfície celular.

Na ausência da enzima lítica do fago, demonstrou-se que LytA medeia uma lise bacteriana extensa acompanhada da libertação de uma grande quantidade de fagos funcionais capazes de completar o ciclo infeccioso, uma vez que foram claramente detectadas placas fágicas. A grande maioria dos fagos que infectam outras espécies bacterianas é absolutamente incapaz de causar lise bacteriana, aprisionando a descendência fágica dentro da célula hospedeira, quando são eliminados os genes que codificam as suas lisinas. Além disso, os poucos mutantes capazes de lisarem as bactérias hospedeiras dependem unicamente de factores codificados por si próprios. No entanto, a dependência exclusiva na autolisina adiou o momento da lise e reduziu a sua extensão. Em concordância, as placas fágicas foram detectadas mais tarde do que as observadas na presença de ambas as lisinas (bacteriana e fágica) e observou-se uma diminuição significativa no número de viriões libertados. Assim, a lise estritamente dependente de LytA pode influenciar negativamente o "fitness" do fago ao reter a descendência fágica durante mais tempo no interior do hospedeiro e ao reduzir a quantidade de partículas que de facto escapam ao aprisionamento. No entanto, em circunstâncias normais de infecção bacteriana, verificou-se que a activação de LytA em simultâneo com a da lisina fágica aumenta o número total de fagos libertos da célula hospedeira uma vez completo o ciclo infeccioso. Logo, os fagos de *S. pneumoniae* utilizam a ubíqua autolisina bacteriana para optimizar a sua estratégia de libertação da descendência sendo, entre os fagos equipados com lisinas, os únicos que dependem de factores líticos bacterianos para a libertação óptima.

Embora as funções da holina e da lisina fágica estejam caracterizadas, a interacção entre estas proteínas para alcançar a lise em *S. pneumoniae* não foi integralmente determinada. Como as lisinas fágicas são estrutural e funcionalmente similares a LytA, é possível que partilhem a mesma localização celular e mecanismos de regulação. A observação de que as lesões na membrana provocadas pelas holinas activam a autolisina bacteriana localizada na parede celular, incitou a um estudo mais detalhado sobre a estratégia de lise do pneumococo.

Para isso, foram eliminadas as funções da holina e da autolisina numa estirpe lisogénica em que o profago contém uma cassete holina-lisina típica. Na ausência destas actividades, a análise por "western blot" e a avaliação na lise das culturas do efeito de agentes que permeabilizam a membrana e dissipam a força motriz protónica (fmp) permitiram concluir que as lisinas fágicas de *S. pneumoniae* acumulam-se ao longo do tempo durante o ciclo lítico e são continuamente transportadas para a parede celular, onde permanecem inactivas associadas aos resíduos de colina. Assim, o acesso das lisinas fágicas à superfície bacteriana é independente das holinas, podendo ser caracterizadas como exolisinas. Estes resultados diferem do que se observa na grande maioria dos fagos dependentes do sistema holina-lisina. Nesses casos, as endolisinas acumulam-se no citoplasma, uma vez que são desprovidas de uma

sequência sinal secretória intrínseca e, consequentemente, dependem das holinas para alcançarem o peptidoglicano. Foi também estudado o envolvimento do sistema Sec do hospedeiro na exportação da lisina fágica. A localização da lisina fágica na parede celular foi avaliada pelos mesmos procedimentos experimentais após tratamento das culturas com azida de sódio, um inibidor desta via de transporte. Demonstrou-se que a enzima lítica fágica é possivelmente exportada pelo sistema Sec de *S. pneumoniae* apesar da ausência de sequências sinal que a possam dirigir para o compartimento extracitoplasmático. Estes resultados podem constituir a primeira evidência experimental, nos fagos que codificam nas suas cassetes líticas apenas as funções de holina e lisina, de uma exolisina sem sinal de exportação que é translocada através da membrana pelo sistema Sec do hospedeiro. Além disso, uma vez que a autolisina bacteriana localizada na parede celular também não apresenta motivos nem sequências de sinalização que justifiquem uma localização externa, estas observações podem ajudar a elucidar o seu mecanismo de transporte.

A lise completa devido exclusivamente ao agente que dissipa a fmp, conjuntamente com a observação anterior do efeito permeabilizante das holinas, demonstrou que o colapso do gradiente electroquímico da membrana citoplasmática provocado pelas holinas constitui o sinal para activar a lisina fágica. Neste estudo, foi também confirmado que a activação da autolisina bacteriana externalizada, que se tinha verificado anteriormente contribuir para a libertação da descendência fágica, está igualmente relacionada com perturbações no estado energético da membrana. Logo, estes resultados demonstram que nos fagos de *S. pneumoniae*, a holina não é necessária para a exportação da lisina mas é crucial para activar tanto a lisina fágica como a bacteriana residentes na parede celular por dissipação da fmp aquando da formação de lesões na membrana, determinando o momento da lise.

Após a caracterização do mecanismo fágico de lise, foi investigada a contribuição da lise mediada por fagos lisogénicos nos biofilmes do pneumococo. Em *S. pneumoniae*, as estirpes lisogénicas estão associadas com a infecção e os biofilmes foram implicados em ambos os processos de colonização e infecção. Foi estudado se a indução espontânea dos profagos e a consequente lise bacteriana favorece o desenvolvimento de biofilmes de *S. pneumoniae* por ser uma fonte de DNA extracelular (eDNA), um factor importante na matriz dos biofilmes.

O desenvolvimento de biofilmes de estirpes lisogénicas e não lisogénicas foi analisado por quantificação da biomassa, determinação da viabilidade celular e por CLSM ("confocal laser scanning microscopy"). Observou-se que as estirpes lisogénicas são mais propensas a formar biofilmes, os quais se caracterizam por maior biomassa e viabilidade celular relacionadas com a maior densidade observada por CLSM. Foi demonstrado que a indução espontânea do fago ocorre continuamente uma vez que se detectaram partículas fágicas durante todo o

desenvolvimento do biofilme por medição do número total de UFPs (unidades formadoras de placas) a tempos específicos. Por comparação da formação do biofilme entre estirpes lisogénicas com e sem a lisina fágica, a autolisina bacteriana ou ambas, foi observado que eventos líticos mediados pelo fago influenciam positivamente a estrutura do biofilme. Estes resultados estabeleceram que o profago promove o desenvolvimento do biofilme através da lise bacteriana aquando da indução espontânea. Em concordância, a lise em biofilmes também ocorre noutras espécies bacterianas e parece estar relacionada com o aumento do crescimento do biofilme.

No entanto, os efeitos da eliminação quer da lisina fágica quer da lisina bacteriana, foram anulados pela adição de DNA. Em experiências independentes, verificou-se que o tratamento com DNase I resultou em biofilmes menos compactos e densos enquanto a suplementação com DNA originou estruturas mais robustas, confirmando o papel importante de eDNA nos biofilmes de *S. pneumoniae*. A quantificação de eDNA presente nestas estruturas por PCR em tempo real também demonstrou que eventos líticos promovidos pelo fago são uma fonte importante deste componente da matriz pois biofilmes de estirpes capazes de lise continham mais eDNA do que as estirpes sem lisinas. Desta forma, a lise limitada mediada pelo fago constitui uma fonte importante de eDNA nos biofilmes de *S. pneumoniae* favorecendo o seu desenvolvimento no caso de estirpes lisogénicas. Interessantemente, a indução substancial do fago, levando a uma elevada proporção de lise, destrói gravemente os biofilmes de estirpes lisogénicas diminuindo significativamente a massa do biofilme como confirmado por CLSM. Estas observações corroboram estudos anteriores que demonstram o potencial do uso de fagos líticos para eliminar biofilmes bacterianos.

Os resultados e conclusões apresentados são importantes não só por alargarem directamente o conhecimento acerca da biologia do fago e da sua relação com o hospedeiro bacteriano, mas em última análise para permitir atribuir à lisogenia um papel claro na virulência do pneumococo. Neste contexto, a indução substancial de lise bacteriana pelo fago pode, tal como a lise promovida pela autolisina LytA, libertar factores que contribuem para o curso da infecção. Por outro lado, a lise devido à indução espontânea característica do estado de profago pode ter um impacto na patogenicidade por influenciar a formação de biofilmes, os quais têm sido implicados nos processos de colonização e infecção. A compreensão dos mecanismos subjacentes à infecção pneumocócica é de extrema relevância para poder controlar este importante agente patogénico.

AIM AND OUTLINE OF THE THESIS

The aim of this study was to identify and characterize factors involved in bacteriophage lysis strategy in the pneumococcal model phage SV1 and subsequently reveal the molecular mechanisms leading to phage release in *S. pneumoniae*. Upon characterization of the phage lytic mechanism, the focus shifted to explore the impact of lysogeny, through phage-mediated lysis, on the bacterial host particularly in the capacity to form biofilms as recent studies demonstrated the potential of *S. pneumoniae* to produce biofilms *in vivo* and propose that pneumococcal biofilms play a relevant role both in colonization and infection.

In **Chapter I**, a detailed overview of the relevant literature introduces and describes the theme. The influence of lysis on *S. pneumoniae* virulence and the lysis strategy of their phages are presented. In addition, current knowledge regarding pneumococcal lysogenic phages as potential contributors to virulence, with special focus on induced host lysis, is reviewed.

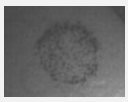
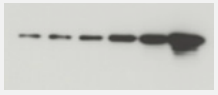
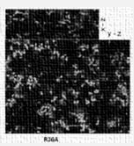
In **Chapter II**, the characterization of *S. pneumoniae* phage-mediated lysis is described, aiming at evaluating the possible role of the bacterial autolysin LytA in phage progeny release. The investigation of the putative control function of phage-encoded holins over both phage and bacterial lysins is also reported.

Chapter III addresses the detailed phage lysis mechanism operating in *S. pneumoniae* with a particular focus on the localization of the lysins. The studies on the precise role of each lysis component of the holin-lysin system and how they work together to accomplish phage progeny release are presented.

The study of the influence of lysogenic phages, through induced lysis, in the host ability to form biofilms is presented in **Chapter IV**. The results comprise mainly the *in vitro* evaluation of the effect of spontaneous induction of the phage lytic cycle on those complex bacterial structures.

The concluding remarks in **Chapter V** provide an integrative framework of the findings presented in this thesis. The relevance of this work and perspectives for further studies are also highlighted in this chapter.

THESIS AT A GLANCE

STUDY	QUESTION	METHODS	MAIN FINDINGS AND CONCLUSIONS
(I) **The autolysin LytA contributes to efficient bacteriophage progeny release in *Streptococcus pneumoniae***  **CHAPTER II**	What is the contribution of the *S. pneumoniae* autolysin LytA to phage progeny release?	Deletions of the bacterial autolysin (LytA) and phage lysin (Svl) were done in *S. pneumoniae* lysogenic strains. Bacterial culture lysis was monitored after MitC phage induction and phage release was estimated by phage plaque assays. Cell membrane integrity during this process was assessed by flow cytometry to study the function of phage holin over lysins activities. *MitC, mitomycin C*	1. The bacterial LytA is activated during the phage lytic cycle mediating host lysis and consequent phage release. 2. LytA activation, just like the phage lysin triggering, is due to holin-induced membrane disruption. 3. Lysis exclusive dependent on LytA, however, impairs phage release that may negatively influence phage fitness. 4. But, activation of LytA concurrent with the phage lysin allows optimal phage release in *S. pneumoniae*.
(II) **Holin-independent export of *Streptococcus pneumoniae* bacteriophage lysins**  **CHAPTER III**	What is the precise holin-lysin lytic mechanism of pneumococcal phages?	Holin and autolysin LytA activities were deleted from *S. pneumoniae* lysogenic strains. The possible cell wall localization of the phage lysin (Svl) was investigated by western blot. The effect of the membrane pmf-dissipating agent DCCD on lysis was tested after phage induction to assess the triggering signal for Svl activation. Host Sec pathway involvement in Svl export was explored using the sec inhibitor NaN_3. After NaN_3 treatment, Svl cell localization was analyzed by western blot and followed up of lysis with DCCD. *Pmf, proton motive force; DCCD,* N,N´-*dicyclohexylcarbodiimide*	1. Pneumococcal phage lysin is targeted to the cell wall in the absence of holin function, where it remains in an inactive form. 2. Lysin export is possibly mediated by the host Sec pathway despite the absence of signal sequences. 3. The phage exolysin is activated by membrane pmf collapse resulting from holin lesions, like the externalized bacterial LytA. 4. In pneumococcal phage lysis strategy, the phage exolysin accesses the cell wall independently of the holin but holins are crucial for phage and bacterial lysins activation.
(III) **Prophage spontaneous activation promotes DNA release enhancing biofilm formation in *Streptococcus pneumoniae***  **CHAPTER IV**	How lysogeny influences pneumococcal biofilm formation? Is host lysis due to phage spontaneous induction an eDNA source that enhances biofilm development?	Biofilm development of lysogenic and nonlysogenic pneumococcal strains was followed by biomass quantification, viable cell counts and CLSM. Phages released during biofilm formation were measured by PFUs. Biofilm development in the absence of the phage lysin and bacterial autolysin LytA was also evaluated. We tested the effect of the addition of external DNA and DNase I and measured the amount of eDNA released within biofilms by real-time PCR. *CLSM, confocal laser scanning microscopy; PFU, plaque forming unit*	1. Lysogenic bacteria are more prone to form biofilms characterized by higher biomass and cell viability. 2. Spontaneous phage induction occurs continuously within the biofilm during its development. 3. Ablation of either the phage lysin or bacterial lysin impairs biofilm development, which is overcome by addition of DNA. 4. Spontaneous phage-mediated host lysis is an important source of eDNA favoring biofilm formation by *S. pneumoniae* lysogenic strains.

LIST OF PUBLICATIONS

The scientific results of this thesis have resulted in the following manuscripts:

Frias, M.J., Melo-Cristino, J., Ramirez, M. 2009. The autolysin LytA contributes to efficient bacteriophage progeny release in *Streptococcus pneumoniae*. J Bacteriol. 191(17):5428-5440.

Carrolo, M.*****, **Frias, M.J.***, Pinto, F.R., Melo-Cristino, J., Ramirez, M. 2010. Prophage spontaneous activation promotes DNA release enhancing biofilm formation in *Streptococcus pneumoniae*. PLoS ONE.5(12):e15678.

* These authors contributed equally to this work.

Frias, M.J., Melo-Cristino, J., Ramirez, M. 2011. Holin-independent export of *Streptococcus pneumoniae* bacteriophage lysins. Submitted to Mol Microb.

LIST OF ABBREVIATIONS

aa	Amino acids
ADP	Adenosine diphosphate
Amp	Ampicillin
APS	Ammonium persulphate
ATP	Adenosine triphosphate
bp	Base pair
C-	Carboxyl (when in polypeptide)
CBD	Choline binding domain
CBP	Choline binding protein
CBR	Choline binding repeat
CFU	Colony forming unit
Cho	Choline
CLSM	Confocal laser scanning microscopy
Cm	Chloramphenicol
CM	Cytoplasmic membrane
CWH	Cell wall hydrolase
DCCD	*N*,*N*´-dicyclohexylcarbodiimide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DOC	Deoxycholate acid
ds	Double stranded
eDNA	Extracellular DNA
EPS	Extracelullar polymeric substances
Ery	Erythromycin
FSC	Forward scatter
GalNAc	*N*-acetylgalactosaminyl
GlcNAc	*N*-acetylglucosamine
h	Hour
IPTG	Isopropyl-β -D-thiogalactopyranoside
kb	Kilo base
kDa	Kilo Dalton
Kn	Kanamycin
LB	Luria Bertani
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MIC	Minimum inhibitory concentration
min	Minute
MitC	Mitomycin C
MLST	Multilocus sequence typing
MOI	Multiplicity of infection
MurNAc	*N*-acetylmuramic acid
MWCO	Molecular weight cutoff
N-	Amino (when in polypeptide)
Nov	Novobiocin
OD	Optical density
Orf	Open reading frame
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis

PBP	Penicillin binding protein
PBS	Phosphate buffer saline
Pce	Phosphorylcholine esterase
PCho	Phosphorylcholine
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pen	Penicillin
PFGE	Pulsed-field gel electrophoresis
PFU	Plaque forming unit
PG	Peptidoglycan
PI	Propidium iodide
Ply	Pneumolysin
Pmf	Proton motive force
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotation per minute
s	Second
SAR	Signal-arrest-release
SDS	Sodium dodecyl sulfate
Sec	Secretion
Sm	Streptomycin
ss	Single stranded
SSC	Side scatter
TA	Teichoic acid
Tris	Tris(hydroxymethyl)aminomethane
TSA	Tryptone soy agar
UV	Ultraviolet
wt	Wild-type
WTA	Cell wall teichoic acid
% (v/v)	Percentage expressed in volume/volume
% (w/v)	Percentage expressed in weight/volume

CHAPTER I

GENERAL INTRODUCTION

1. *STREPTOCOCCUS PNEUMONIAE*

Streptococcus pneumoniae, or pneumococcus, causes a wide variety of human infections ranging from potential fatal septicaemia, meningitis and pneumonia to more uncomplicated conditions including otitis media, sinusitis and bronchitis [1]. In most instances, *S. pneumoniae* will not cause infection in humans and can simply be carried by the host, colonizing the upper respiratory tract. Carriage of the bacterium is, however, believed to be the first step in the pathogenesis of this organism [2,3].

The treatment of pneumococcal infections is done with antibiotics, although they remain associated with high morbidity and mortality [4]. With the increasing prevalence of antibiotic resistant strains [5], a strategy of prevention by vaccination has been developed to counter the problem. Current vaccinations target the capsular polysaccharide that coats *S. pneumoniae*. However, over 90 different chemical variants of this polysaccharide exist within the species [6-9], and all the available vaccines only protect against a few of the capsular types (serotypes) [2,3]. Alternative vaccination approaches using other components of the pneumococcal surface are currently being studied but issues in their development include lack of immunogenicity or variability in different strains [2,3].

Considering these growing difficulties it is crucial to design novel therapeutic regimes which imply better knowledge of the factors and processes implicated in the virulence of this species.

2. BACTERIOPHAGES OF PNEUMOCOCCUS

Bacterial viruses or bacteriophages (phages) are profoundly involved in bacterial pathogenesis. They are essential vehicles for the transmission of virulence genes within bacterial populations [10-14]. Taking into account the abundant presence of lysogenic phages (that integrate its genome into the bacterial chromosome) among *S. pneumoniae* strains associated with infection (76%) [15], pneumococcal phages have been studied to understand their putative impact in virulence.

2.1. GENERAL CHARACTERISTICS

Pneumococcal phages were first isolated in 1975 from throat swabs of healthy children [16,17]. Since then, several phages of this bacterium from different origins have been isolated and characterized [18,19]. They have, like most phages, double stranded DNAs encapsulated

into a protective head (capsid) attached to a tail [18-23], and comprise three families, belonging to the *Caudovirales* order (tailed phages), with varied morphologies (Fig.I.1) [18,24]. Dp-1, Dp-4, ω, HB-3, MM1 e VO1 phages belong to the family *Siphoviridae* and have long noncontractile tails [18,21,22,25,26], whereas EJ-1 is classified as *Myoviridae* with a long contractile tail [27]. On the other hand, Cp phages (Cp-1, Cp-5, Cp-7 and Cp-9) have a short noncontractile tail and belong to *Podoviridae* (Fig.I.1) [18,19]. Also, the SV1 phage, identified in *S. pneumoniae* strain SVMC28 responsible for human infection, has a typical viral morphology of the *Siphoviridae* family and is very similar to HB-3 (Fig.I.2) [15].

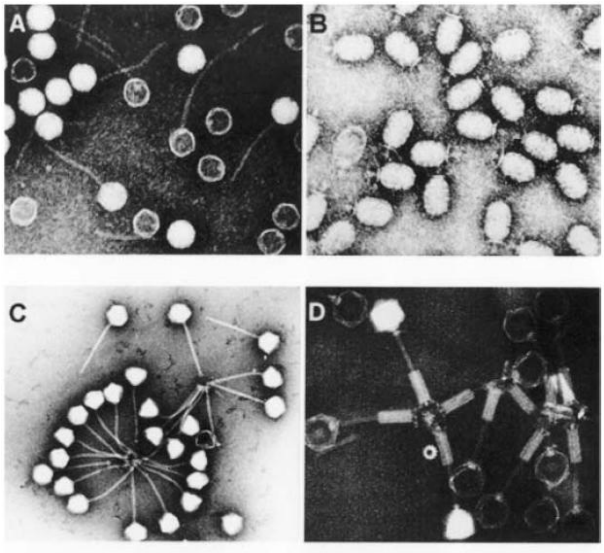


Figure I.1. Electron micrographs of purified pneumococcal phages. (**A**) Dp-1; (**B**) Cp-1; (**C**) HB-3; (**D**) EJ-1. Electron microscopy magnifications are not indicated. From [18].

Generally, their host range is restricted to *S. pneumoniae*, that is, they infect specifically pneumococcal cells [18]. Cp-1 phage represents an exception because it can infect and replicate in *Streptococcus oralis*, which is taxonomically related to pneumococci and shares a common habitat in humans [28]. Pneumococcal phages may also infect specific strains within this species [18]. For instance, EJ-1 was purified from cultures of the pneumococcal strain 101/87, but repeated attempts to infect *S. oralis* or even several pneumococcal strains with EJ-1 were unsuccessful [27].

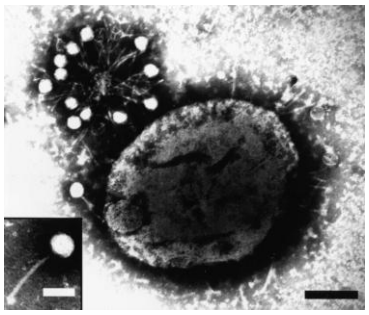


Figure I.2. SV1 phage particles of pneumococcal strain SVMC28. The larger electron micrograph shows a pneumococcal cell with multiple phage tails attached and a cluster of phages and phage tails in the top left corner. The inset shows a complete SV1 phage. Black bar, 0.3 µm; white bar (inset), 0.1 µm. From [15].

2.2. LIFE CYCLE

Phages, like all other viruses, are obligate intracellular parasites and thus, in order to replicate they require a specific host. In the extracellular environment, phage particles are just inert packages that protect the viral genome from damaging environmental factors until the vital nucleic acid is delivered into a susceptible host [23]. Pneumococcal infection by phages can lead to either of two responses, the lytic or the lysogenic cycle [18,24].

2.2.1. LYTIC PHAGE INFECTION

The phage vegetative growth or lytic pathway starts with phage attachment or adsorption to the bacterium (Fig.I.3) [23]. Phage tails are known to interact with receptors present on the bacterial surface [23]. The majority of phages are highly specific for their host receptors [23]. In *S. pneumoniae*, adsorption of phage Dp-1 was shown to be dependent on choline, a structural component of the teichoic acids of the pneumococcal cell wall [29]. In fact, a protein contained in Dp-1 tail, besides binding to other molecules, also attaches to choline residues [20]. A detailed analysis of the open reading frame (orf) encoding such a protein identified a motif similar in amino acid sequence to that characteristic of the choline binding domain of pneumococcal proteins [20]. Curiously, four repeats of this motif were found [20], which appears to be the minimum required for efficient binding of the protein LytA (section 3.3) [30].

After adsorption, phage genetic material penetrates into the host cell with the capsid remaining externally (Fig.I.3) [23]. Once inside the host, phage gene expression and genome replication begin (Fig.I.3). Phage gene expression generally occurs in different stages [23]. At the initial phase of infection, genes involved in genome replication are expressed (“early genes”). Only afterwards, occurs the expression of the genes encoding structural components of the phage particles and factors involved in their assembly (“late genes”) [23]. This temporal synthesis has been demonstrated in pneumococcal Cp-1 phage and apparently occurs similarly

in all phages of this bacterium [31,32]. Synthesis of viral structural proteins usually overlaps with the nucleic acid replication. The new virion building material accumulates within the cell and when there are plenty of replicated genomes and structural proteins available, assembly of new virions occurs with packaging of the genomes into the phage particles (Fig.I.3) [23].

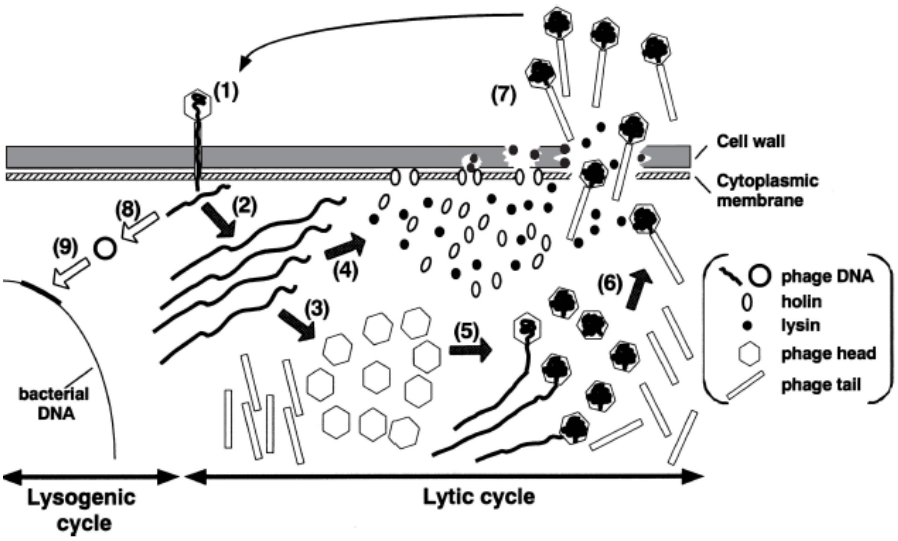


Figure I.3. Phage lytic and lysogenic life cycles. Phage infection starts with adsorption to the cell surface receptor and genome penetration into the host (1). In the lytic infection, it follows genome replication (2), head and tail production (3), synthesis of lysis proteins (4), genome packing (5) and phage particles assembly (6). In the end of the lytic cycle, disruption of the cell and release of the phage progeny occurs (7). In the lysogenic cycle, after genome penetration, phage genome is circularized (8) and integrated into the host genome (9). From [33].

Finally, the newly formed and fully assembled virions exit the host by cell lysis (Fig.I.3) [18,20,23]. Proteins involved in phage release are also expressed late in infection by phage Cp-1 [31]. Besides Cp-1, pneumococcal phages Dp-1, Dp-4 and Cp-7, replicate exclusively by vegetative growth and therefore are classified as virulent or lytic [18,20,24] since infection is shortly followed by their bacterial host death and lysis.

2.2.2. LYSOGENY AND PROPHAGE INDUCTION

In the lysogenic phage infection (lysogeny), the phage genome can be integrated into the chromosome of the bacteria and replicated along with the host genome, existing in a latent prophage state (Fig.I.3) [34,35]. This arrangement offers the virus an alternative mechanism for making more copies of its genome. Bacteria that carry prophage are said to be lysogenic (or lysogens) and generally became resistant to infection by other related phages. Phages that can undergo lysogeny, such as MM1, HB-3, VO1, EJ-1 and SV1 of *S. pneumoniae* [15,18,25,36,37],

are called temperate or lysogenic. However, the mechanisms leading to the decision, soon after phage genome penetration, whether to follow the lytic or the lysogenic pathway remain largely unknown [34,35].

The phage genome integration occurs by a site-specific recombination process [38]. Two specific attachments sites, one on the bacterial chromosome (*attB,* bacterial attachment site) and the other on the phage genome (*attP*, phage attachment site), are recombined by the activity of a phage-encoded integrase. Although the integration system of pneumococcal phages is poorly documented, the *attP*-containing phage DNA, the bacterial attachment site *attB* or the host-phage junctions *attL* and *attR* have already been identified in some prophages such as MM1, EJ-1 and VO1 [25,26,37]. Even more, in the surrounding region of the *attP* of EJ-1, there are nucleotide sequences that share similarity with a sequence that was suggested to participate in the binding of integrases [18].

During lysogeny, the expression of phage genes whose products are required for the lytic pathway is prevented by the action of the viral repressor (CI) [34,35]. Although the prophage state is relatively stable, it can be induced to enter the lytic growth leading to the production of progeny virions, through the host machinery, and cell lysis [34,35]. At high levels of prophage induction in a lysogenic culture, triggered by external events, lysis can occur in all or nearly all of the cells [35]. In addition, even in the absence of an inducer, spontaneous induction occurs, although at a lower level, resulting in the lysis of a small fraction of the cells [35,39-41].

In the *Escherichia coli* phage λ, the mechanism of prophage induction is well characterized. It is triggered by treatments that damage DNA and inhibit DNA replication. This elicits the action of the host SOS regulatory system (SOS response), which involves the action of proteins LexA and RecA, to repair the DNA and restore replication [34,35]. Upon inducing treatments, RecA is activated and catalyzes the proteolytic self-cleavage of LexA. Since during normal cell growth LexA represses a large set of genes (SOS genes) normally with a basal level of expression, its cleavage inactivates the repressor function and the SOS genes are expressed at high levels, including *recA*. In parallel, activated RecA also promotes the cleavage of CI phage repressor, leading to derepression of the prophage (which causes the phage to excise from the host chromosome and enter the lytic cycle) [35]. When the damaged is repaired, RecA is no longer activated and LexA builds up rapidly to normal levels that again ensure repression of the SOS genes. It should be noted that, in the λ system, spontaneous switching still requires the activation of SOS response [35] nonetheless, even in this well-studied phage, the factors that lead to this spontaneous process are poorly understood.

Since the first isolation of lysogenic phages from *S. pneumoniae* [42], lysogeny was found to be very frequent among pneumococcal strains recover from human infections [15,43] (with as much as 61% of strains carrying functional prophages [15]). Although it was initially suggested that lysogeny was associated with only certain pneumococcal capsular types causing disease [44], the presence of fully functional, defective (noninducible) or remnant prophages in the chromosome is indeed a general trait among *S. pneumoniae* isolates [15,26], including nonencapsulated pneumococci [27]. Despite this widespread presence, prophage induction mechanisms are still little studied. However, in the lysogenic cluster of phage MM1, it was recently demonstrated that a protein with a putative repressor function (repressor CI) [36] is indeed involved in the maintenance of the lysogenic state [45]. Moreover, the treatment with mitomycin C (MitC), which induces the lytic growth from the prophage state in lysogenic *E. coli* [46] since it crosslinks the complementary strands of the DNA double helix [47] blocking the replication fork, was also proven to induce pneumococcal prophages [15]. In MitC-treated pneumococci the presence of phage particles inside the cell were visualized just before lysis occurred (Fig.I.4). Another interesting characteristic shared with the *E. coli* system is the effect of MitC, at nonbactericidal concentrations used to induce prophages, on cell elongation as a result of a block in cell division with a concomitant increase in cell mass (Fig.I.4) [15,46,48]. Furthermore, even though *S. pneumoniae* lacks a canonical SOS system, a RecA protein was identified as being involved in DNA repair [49]. It was shown that RecA controls directly or indirectly lysogenic induction since MitC treatment results in increased *recA* expression and in the absence of RecA there is no prophage induction and cell lysis [49,50].

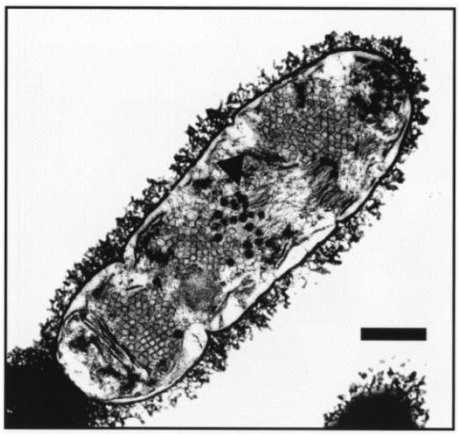


Figure I.4. Intracellular SV1 phage particles in *S. pneumoniae* strain SVMC28 induced with mitomycin C. Lysis was prevented by a high concentration of choline in the medium. Similar observations were obtained with MitC-induced cells just before lysis, without choline treatment. Collapse of the membrane is evident from the white background in the cytoplasmic space. Escape of phage particles is prevented by the integrity of the cell wall. Arrowheads indicate fully assembled phage particles. Bar, 0.3 mm. From [15].

2.3. PHAGE LYSIS STRATEGY

Phages use two basic strategies to escape from the infected cell [51-53]. All known phages release their progeny by lysis of the host, except the filamentous phages, such as M13, that can extrude themselves through the host envelope without fatal consequences to the bacteria [23]. To lyse the host, phages have to compromise the bacterial cell wall [51-53]. Small phages with single stranded (ss) genomes (ssDNA and ssRNA) encode a single lysis protein (without cell wall hydrolyzing activity) that typically inhibits a specific step in cell wall synthesis during active bacterial growth [51-54]. Since cell wall synthesis is coupled with turnover and recycling of the preexistent cell wall (see section 3.1), the action of the phage protein weakens the cell wall and eventually results in cell rupture due to the osmotic pressure difference between the internal and external environments. The E protein of ssDNA phage ϕX174 of *E. coli* is an example of this strategy [54]. On the other hand, dsDNA phages, that correspond to the great majority of phages (about 96%) [23], generally encode at least two proteins that together promote cell wall degradation accomplishing rapid cell lysis, known as the holin-lysin system [51-53,55].

2.3.1. HOLIN-LYSIN SYSTEM

In the holin-lysin lysis strategy, the lysin is a cell wall hydrolyzing enzyme and the holin is a lethal protein with transmembrane domains that permeabilizes (making holes) the cytoplasmic membrane [51,56-59]. On both Gram-positive and Gram-negative hosts, the phage lysin is generally deprived of a secretory signal sequence hence it is sequestered in the cytoplasm and designated endolysin. Therefore, it requires the holin to gain access to its cell wall substrate (Fig.I.5A) [51-53,56,57,60]. In fact, it was observed for some phages that in the absence of holin function cell lysis is prevented but endolysin activity accumulates in the cell with no deleterious consequence on the growth despite being enzymatically active [61,62]. Accordingly, cell culture mass and intracellular phage titers continue to build up for an extent period of time [61-63]. When the endolysin function is impaired, in the presence of holin, lysis is also abolished. However, in contrast to what happens without holin, cell death (culture viability loss) occurs at the normal time of lysis and macromolecular synthesis stops with no further increase in biomass and virion production [57,61,63,64]. Thus, the holin permeabilizing function determines the timing of lysis [56].

It was shown that the precise moment of lysis is critical for phage fitness [65]. On one hand, if lysis occurs too early in the infection cycle few progeny virions are released. Delay of

lysis, on the other hand, prevents progeny virions from infecting new hosts, despite producing more progeny [65]. It is therefore understandable that host lysis, hence holin activity, must be a fine-tuned event [56]. Based essentially on *E. coli* phage λ studies, a model for lysis regulation was proposed [66]. During the late stage of phage development holins progressively accumulate inactive in the membrane without detrimental effects in membrane integrity [67,68]. Meanwhile, these small proteins dimerize and then assemble into oligomers eventually leading to the formation of protein aggregates or rafts, largely excluding lipid from the interior [66,69,70]. At a specifically programmed time during the growth of a raft, the protein accumulation would result in protein-protein interactions weakening, which would cause the opening of a momentary channel or hole (local membrane disruption) in the protein array. This leads to an instantaneous local depolarization of the membrane which in turn causes conformational changes in the holins that further destabilize the interactions, triggering rapidly lesion formation (massive membrane disruption) [66,70]. Consequently, sudden global membrane proton motive force (pmf) collapse occurs only seconds before lysis (Fig.I.6) [67]. Recently, it was observed that λ holins accumulate in a uniformly distributed fashion in the membrane and raft formation (redistribution into holin aggregates) only occurs at the time of holin triggering [71]. In this case, it was proposed that the holin rafts form suddenly when the holin reaches a critical concentration and are inherently incapable of supporting the membrane potential, likely because the raft interior is lipid depleted, leading to the local depolarization [71]. Generally, the holin primary structure of a specific phage was implicated in the decision of the lysis timing, i.e. the moment of the first disturbance in the protein array, in accordance with the observation that different phages exhibit different lysis times [56,69,72]. In the overall process, the energized membrane is essential for holin control preventing deleterious anticipation of membrane disruption and guaranteeing that phage production within the cell is not compromised until just the optimal moment of release [67]. This can explain the well-known effect of premature lysis triggering by energy poisons during the period when holin accumulates in the membrane [52,56,67].

Additional means to achieve a precise lysis timing, at least in some cases, rely on the synthesis of a holin inhibitor [68,73-75]. In phage λ, the antiholin was shown to directly interact with the holin probably resulting in mixed arrangements of holins and antiholins accumulated in the cytoplasmic membrane that are nonfunctional for hole formation [68,76]. As long as membrane potential is maintained, antiholin delays hole formation by the holin since accumulation of the holin functional arrangements to trigger the local disruption of the membrane simply takes more time. However, upon dissipation of membrane potential, the

antiholin no longer functions as a lysis inhibitor since its activity is converted into active holin, contributing to the effective formation of lesions [51,56,68,76].

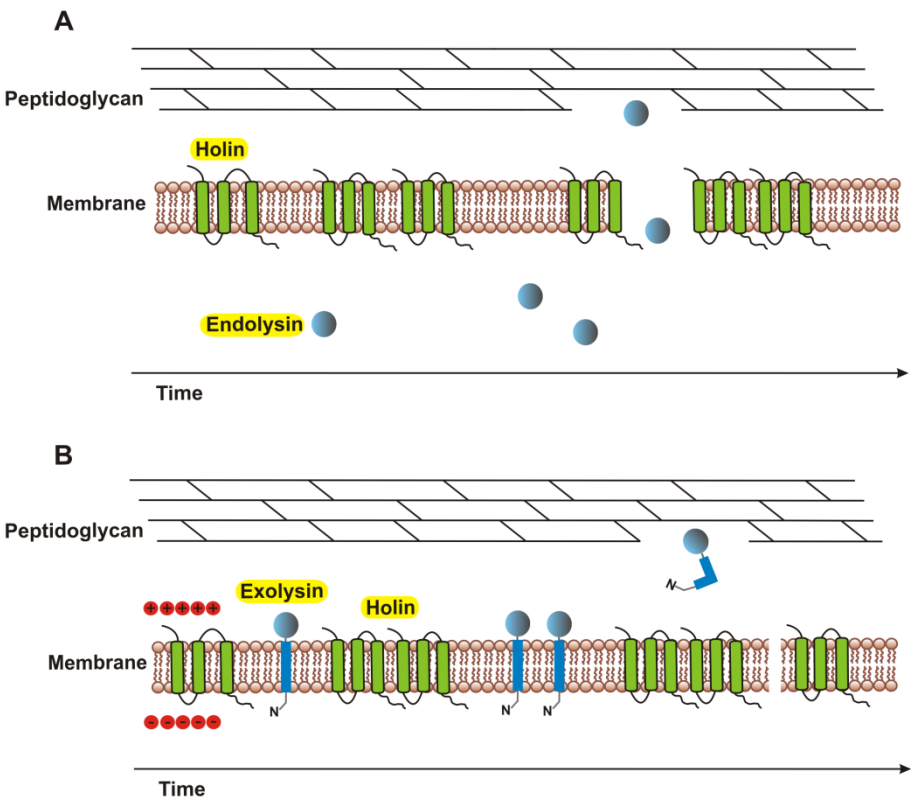


Figure I.5. **Holin-lysin system for triggering of lysis.** (**A**) Classical model for endolysin-equipped phages, based on phage λ. (**B**) New model for phages encoding exolysins, based on the SAR lysin of phage P1 attached to the membrane by its SAR domain. Adapted from [77].

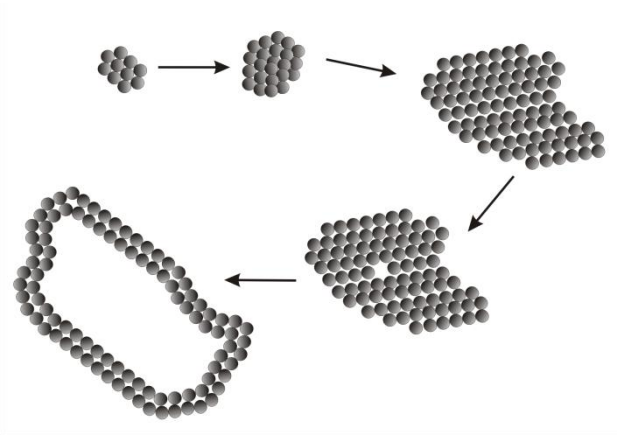


Figure I.6. Original proposed model for the formation of a holin lesion. Holins progressively accumulate in the cell membrane, oligomerize and ultimately form large protein aggregates ("death rafts") from which lipids are largely excluded by intimate interaction between individual holins via their transmembrane domains. Each circle represents a single holin molecule. Spontaneous opening of a local hole at some point in the growth of the raft with consequent localized depolarization of the membrane causes conformational changes in the holins and subsequent dispersion of the subunits into the holin lesion in the membrane. This results in generalized membrane pmf collapse before lysis. From [66].

Regardless of the mechanisms involved in lesion formation, in some endolysin-equipped phages, the holin-induced lesions have actually been shown to be large enough for fully folded lytic enzymes to pass through the membrane into the extracytoplasmic environment [51,56,57,66], supporting the classical paradigm of holin-dependent transport of the endolysin. However, recently, experimental evidence arose opposing to the common belief that intracellular accumulation of phage lysins is a universal feature of phage lysis systems. A phage lysin with a signal peptide (called exolysin) was first found in fOg44 lysogenic phage of the Gram-positive bacterium *Oenococcus oeni* [78]. The lysin Lys44 export to the extracytoplasmic environment does not require the holin action since the typical cleavable N-terminal signal sequence allows host Sec-mediated membrane translocation as phage infection progresses [78]. A surprising observation was the fact that the phage also encodes a holin, Hol44 [79]. Treatment with nisin, which permeabilizes the cytoplasmic membrane (forms 2.5 nm pores) mimicking holin-induced disruption, triggered Lys44-mediated lysis. Pmf-dissipating agents also elicited a lysis response, although not as effectively as nisin [79]. Thus, it was suggested that membrane pmf dissipation due to holin permeabilizing lesions is necessary for lysin activation. However, based on these results the authors also proposed that pmf perturbations are not sufficient to trigger the full activity of Lys44 [79].

Phage lysins with intrinsic export signals were also identified in Gram-negative bacteria [77,80,81]. The lysins of *E. coli* phages P1 and 21 have an atypical SAR (signal-arrest-release) sequence in the N-terminal domain that mediates their translocation across the membrane into the periplasm through the host Sec machinery without proteolytic cleavage. In the extracytoplasmic compartment, the lysin accumulates anchored to the energized membrane in an inactive form by its N-terminal (transmembrane) SAR domain (Fig.I.5B) [77,80,81]. Again, lysin access to the cell wall is holin independent. However, similarly to fOg44 phage, the holin function is still required for cell lysis [77,79-81]. The collapse of the membrane pmf upon formation of holin lesions detaches the SAR domain and consequently activates the lysin, then available in the periplasm enzymatically active to degrade the cell wall (Fig.I.5B) [77,80-83]. Furthermore, the phage 21 holin was shown form small holes (pinholes) in the membrane, in contrast to the large holes formed by λ holins that allow release of fully folded proteins [66], supporting its role of only bringing about membrane depolarization [81,84]. Indeed, this pinholin was not able to function with the λ lysin in inducing cell lysis, i.e. its lesions do not allow the passage of this lysin [81]. Holins do not specifically interact with the endolysins and most holins tend to complement λ holin mutants [56,57]. Apparently, phage 21 holin only promotes lysis when paired with SAR lysins [81]. Interestingly, in both 21 and P1 systems, without holin function phage plaque formation and culture lysis could also be observed,

although the latter characterized by a gradual nature [77,81]. Thus, holin function is not strictly essential for phage release to take place as SAR lysins are apparently detached spontaneously from the membrane albeit at a lower rate [77,81].

Furthermore, it was revealed, mostly by sequence comparisons, that other phage lysins from Gram-positive and Gram-negative bacteria also have N-terminal export sequences, which possibly explore the Sec system [55,85-87]. One peculiar case is the mycobacteriophage Ms6 in which the lysin LysA transport also occurs in a holin-independent manner. Although no export signal was associated with its lysin, this phage additionally encodes a chaperone-like protein (Gp1) in its unusually complex lytic cassette that interacts with the phage lysin and this interaction is necessary for LysA delivery to the cell wall [88]. When tested in *E. coli*, the Sec system was found to be also involved in Gp1-assisted export of the mycobacteriophage lysin [88]. A depolarizing role of the holins in LysA activation was also suggested using permeabilizing drugs [88]. While the holin Gp4 of Ms6 has a N-terminal SAR domain, a characteristic described for the phage 21 pinholin [82,84], it was able, like the canonical P1 holin, to promote the release of the λ lysin [81,89]. With both classical and new holin-lysin models in mind (Fig.I.5A and B), it is clear that there are two different mechanisms by which holins can control lysis timing but both can be viewed as activation of the phage lysins.

2.4. HOLIN-LYSIN STRATEGY OF *S. PNEUMONIAE* PHAGES

In the pneumococcal system there is evidence that the holin-lysin strategy is present in every phage analyzed so far [20,24,25,36,90,91]. The holin and lysin genes, like in the majority of phages described, are located adjacent to each other and clustered in a lytic cassette that may also encompass an antiholin function [20,24,36,90,91]. For instance, the MM1 phage contains two genes, *orf50* and *orf51*, just upstream of the lytic gene, which encode two proteins (138 and 110 amino acids, respectively) that display the characteristic sequence signatures of holins (gp50) or antiholins (gp51), although the actual roles of these proteins in the host lysis machinery are still uncertain [24,36]. For the lytic Cp-1 and lysogenic EJ-1 phages, a detailed characterization of the lysis system was undertaken by cloning and expression of the two genes involved in the release of phage progeny [90,91].

Phage Cp-1 encodes a holin (Cph-1) of 134 amino acids (14.5 kDa) with 3 potential hydrophobic transmembrane regions and the *cph1* gene is found upstream of the *cpl1* gene encoding the lysin Cpl-1 [91]. A antiholin function is not enclosed in its lytic cassette [91]. The cloning and expression of the holin in *E. coli* heterologous system did not induce lysis but dramatically affected cell viability, whereas the concomitant expression of both holin and lysin

resulted in cell lysis and the viability loss was similar to that observed with the holin alone [91]. Moreover, the single expression of lysin did not affect either growth or cell viability. In an *S. pneumoniae* mutant deleted in the major autolysin, the expression of *cph1* and *cpl1* genes had the same effect as in *E. coli* cells, inducing culture lysis and cell viability loss to a similar extent [91]. In addition, the holin itself did not lyse the culture nevertheless, there was no noticeable reduction in cell viability besides the observation of a reasonable impairment in the culture growth rate [91]. Again, pneumococcal lysis was never observed in the presence of Cpl-1 alone before the normal time of lysis [91,92]. From these results it was clear that the phage lysin is activated through holin function [91]. Since the Cpl-1 lysin does not exhibit a secretory signal sequence that could target it to host translocation systems [20], and the viability loss as well as the growth inhibition induced by the holin is compatible with the formation of membrane lesions, it was suggested that, similarly to the majority of holin-endolysin equipped phages, Cp-1 holin might disrupt the cytoplasmic membrane to release the lysin onto its cell wall substrate. The observation that Cp-1 holin complemented the λ phage lysin in producing phage plaques, indicative that it functionally replaced the λ holin in allowing the lysin passage to the cell wall, seemed to corroborate this hypothesis [91].

Similar to Cp-1 phage, EJ-1 phage simply encodes a holin (Ejh), of 85 amino acid residues with two hydrophobic regions, and a lysin (Ejl) with no signal sequence, in which the *ejh* gene precedes the *ejl* lysin gene in the lytic cassette [90]. In both Gram-negative bacteria *E. coli* and *Pseudomonas putida*, cell lysis was only induced by the combined expression of *ejh* and *ejl* while expression of the *ejh* gene resulted in reduced viability attributed to the formation of lesions in the cytoplasmic membrane, as detected by electron microscopy [90]. In agreement, EJ-1 phage holin was also capable of efficiently complementing phage λ lysin to produce phage progeny and an immediately lysis by Ejl was observed after addition of chloroform, that makes membranes permeable [90]. Additionally, for both Cp-1 and EJ-1, it was observed the lysis of *E. coli* cells expressing concomitantly the phage holin and the bacterial major autolysin LytA that is functionally similar to pneumococcal phage lysins (see section 3.3).

Recently, a deeper insight into the function of Ejh was achieved [93]. Ejh is translated as a single polypeptide chain that inserts into the membrane and folds as a helical hairpin directing both terminal ends towards the cytoplasmic face. Upon a given concentration of monomers in the membrane it readily oligomerizes. The self-assembling tendency correlates with the formation of transmembrane holes (Fig.I.7) [24,93]. The hole size allows the exchange of components between the cytoplasm and the external environment, passage of dextrans of various sizes and is sufficiently large to allow for the escape of its 36 kDa Ejl lysin partner [24,93].

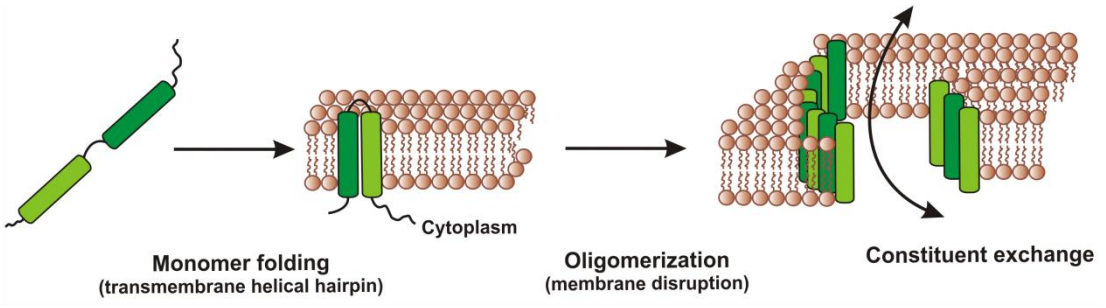


Figure I.7. Model for Ejh holin function. Putative transmembrane regions of Ejh are depicted as green cylinders. From [24].

Based essentially on the nature of the Cph-1 and Ejh holin lesions, large enough for lysins to pass through, and considering that, not only Cp-1 and EJ-1 lysins but all pneumococcal phage lysins lack any known secretory signal sequence (see also Fig.I.12) [20], it is assumed that the holin-dependent delivery of the lysin to its target is a widespread strategy among *S. pneumoniae* phages.

It should be mentioned that long before the holin-lysin system was identified in *S. pneumoniae*, a possible role of the host autolytic enzyme LytA in the release of phage progeny from Dp-1 infected pneumococci was proposed [94,95]. Nevertheless, those studies were not conclusive mainly because the existence of phage-encoded lysins had just begun to be unravelled [95] and the majority of the conditions used to specifically address the function of the host LytA most likely interfered with that of the phage lysin as well [94,95], since pneumococcal phage and bacterial lytic enzymes have vastly common structural and functional features [20].

Given these exceptionally similarities, further insights into the localization, structure and biological role of the bacterial autolysin will be presented, as well as an overview of the shared properties of the *S. pneumoniae* phage and bacterial lysins.

3. PNEUMOCOCCAL PHAGE AND BACTERIAL LYSINS

3.1. CELL WALL

The cell envelope of the pneumococcus, a Gram-positive bacterium, consists of a cytoplasmic membrane and a thick outer cell wall lying under the bacterial capsule. Being the physical surface of the bacteria the cell wall serves as the anchor for capsular polysaccharides (generally covalently bound although the site and nature of the bond remains elusive) as well as for a variety of surface proteins [96,97]. It maintains the integrity of the cell against the

osmotic pressure. Despite being the major determinant for cell shape the cell wall still has to retain certain flexibility since it is involved in key physiological processes such as bacterial growth and cell division.

The cell wall is mainly composed of peptidoglycan (PG), of which the basic structural components are long glycan strands (polysaccharides) consisting of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues, bound by β-1,4 linkages, and small peptides (tri, tetra and pentapeptides) (Fig.I.8) [97-99]. The peptides are linked by the N-terminal region to the glycan strands through the carboxylic group of MurNAc and consist of alternating L and D amino acids, with a pentapeptide corresponding to the sequence L-alanine, D-isoglutamine, L-lysine and two consecutive C-terminal D-alanine residues (L-Ala – D-iGln – L-Lys – D-Ala– D-Ala). Adjacent glycan strands can be crosslinked via peptides bound (by the L-lysine of a peptide with the D-alanine of another peptide) directly or by short dipeptide bridges containing L-serine and/or L-alanine (L-Ser – L-Ala or L-Ala – L-Ala) (Fig.I.8 and I.10) [97-99]. Generally, the composition and degree of crosslinking seem to be highly specific of each *S. pneumoniae* strain [97,99,100]. For instance, a preponderance of indirectly crosslinked components was demonstrated in several penicillin-resistant clinical isolates [99], with the penicillin-resistance of strain Pen6 highly correlated to the preferential incorporation of the seryl-alanine dipeptide bridges [101].

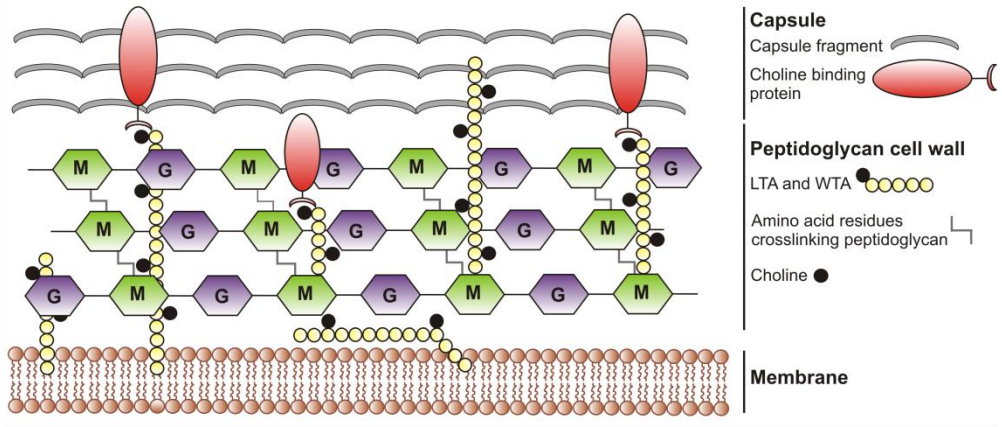


Figure I.8. Structure of the pneumococcal cell wall. The cell wall is built up by several layers of peptidoglycan, forming a dense network. Other constituents include cell wall teichoic acids (WTA) and lipoteichoic acids (LTA), proteins and polysaccharides. Peptidoglycan strands consist of alternating *N*-acetylglucosamine (G) and *N*-acetylmuramic acid (M) molecules and are linked to each other through peptides (attached to M) connected directly or via short dipeptide bridges. WTAs are attached to the M residues by an unknown linkage and LTAs are bound to the cytoplasmic membrane. Choline binding proteins bind to the choline residues of TAs.

The biosynthesis of the peptidoglycan starts at the cytoplasm with the intracellular synthesis of lipid II, the linear muropeptide precursor (GlcNAc-MurNAc-pentapeptide) anchored on the membrane through a carrier lipid, followed by its processing with the addition of L-Ser – L-Ala or L-Ala – L-Ala dipeptide branch to the L-lysine of the muropeptide (Fig.I.9). The completed precursors are then transferred from the cytoplasm across the membrane onto the bacterial surface and extracellularly assembled to form the macromolecular PG. The polymerization of the glycan strands is mediated by a transglycosylation reaction and the covalent linkage of peptides of close proximity among neighbouring glycan strands (between the D-alanine of one peptide and the L-lysine of another) by a transpeptidation reaction (Fig.I.9). The transglycosylation and transpeptidation biosynthetic reactions are carried out mainly by the penicillin binding proteins (PBPs), that are also the major targets of β-lactam antibiotics [97,102,103]. Expression of low affinity derivatives of these PBPs confers penicillin resistance to the bacteria [97].

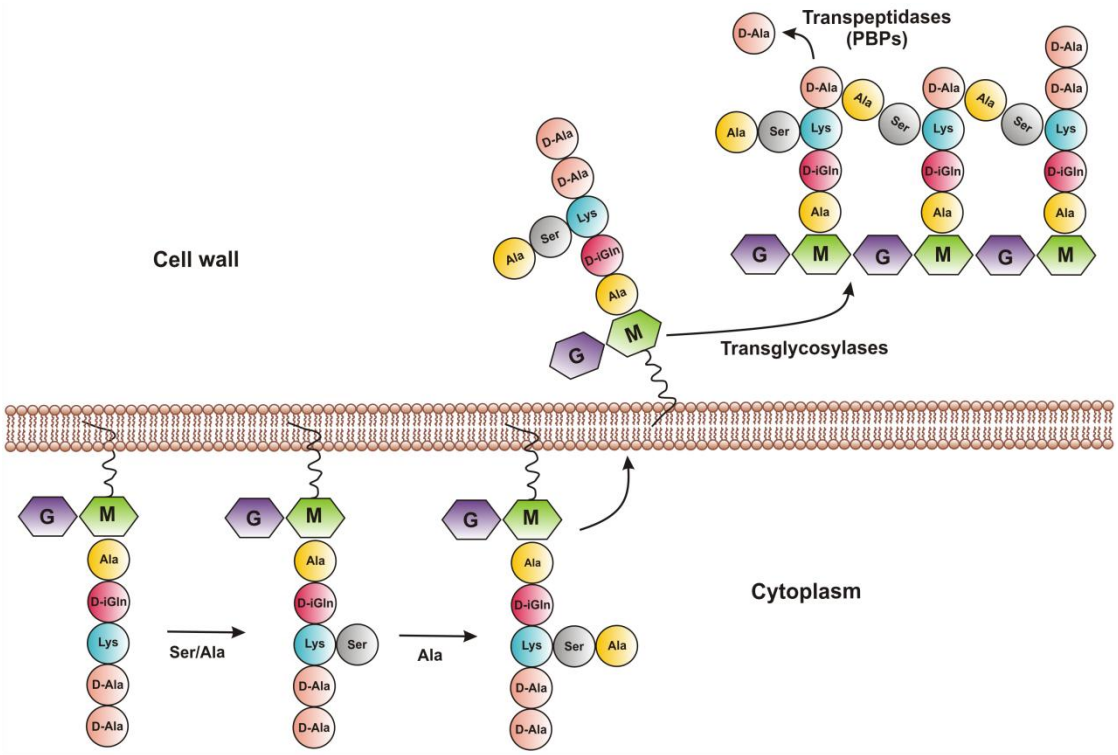


Figure. I.9. Peptidoglycan synthesis. Lipid II is composed of *N*-acetylated disaccharide units of *N*-acetylglucosamine (G) and *N*-acetylmuramic acid (M) with the pentapeptide chain attached to the M residues. Lipid II is anchored on the cytoplasmic membrane through a carrier lipid (ziz-zag line). The addition of a L-serine or L-alanine residue to the L-lysine in the pentapeptide is followed by the addition of an L-alanine to complete the dipeptide branch. Attachment of the completed precursor to the preexisting cell wall occurs on the outer surface of the cytoplasmic membrane by the activity of transglycosylases and transpeptidases. Adapted from [97].

Another important group of surface polysaccharides, embed in the cell wall of all pneumococci, comprises the structurally related cell wall-bound teichoic acid (WTA) and the membrane-associated lipoteichoic acid (LTA) (Fig.I.8) [97,98,104]. The teichoic acid (TA) backbone is common to both WTAs and LTAs and consists basically of a repeating unit that includes two *N*-acetylgalactosaminyl residues (GalNAc) [104]. Due to their characteristic attachment sites on the pneumococcal surface the two teichoic acid derivatives only differ in one structural property: while WTA is covalently linked by an unknown linkage unit to the MurNAc residues of the peptidoglycan, the incorporation of LTA into the bacterial membrane is achieved through a terminal lipid-anchor [97,104]. As they are negatively charged, TAs are partially responsible for the negative charge of the cell surface [105].

A characteristic feature of *S. pneumoniae* is the unusual presence of the aminoalcohol choline in its cell wall [106]. Choline is incorporated as phosphorylcholine (PCho) into WTA and LTA [98,104,107,108] and, depending on the particular *S. pneumoniae* strain, up to two PCho residues can be covalently linked, via GalNAc residues, per repeating unit of the teichoic acid backbone [104,109,110]. The biosynthesis of cholinated teichoic acids is a cooperative interplay of several parallel enzymatic reactions only partially known. While the teichoic acid precursor backbone is intracellularly assembled, choline has to be taken up from the extracellular environment, processed within the cell and ligated, as PCho, to the teichoic acid precursor backbone, apparently before the cholinated teichoic acid can be flipped across the membrane and connected to the peptidoglycan scaffold of the cell wall [97,111]. It was proposed that teichoic acid precursors without choline moieties are not transferred to the outer surface of the membrane [111]. Pneumococci are not able to synthesize choline and a potential exogenous source in the *in vivo* environment could be the degradation of phospholipids by appropriate enzymes to free choline, since phosphorylcholine does not fulfil the nutritional requirement of *S. pneumoniae* [97,112].

Although choline can also be found on the surface of few other respiratory tract pathogens, including streptococcal species (e.g. *S. oralis* and *S. mitis*) [113-115], *S. pneumoniae* has a unique dependence on choline for growth [97,112,115]. It was suggested that the biosynthesis of peptidoglycan and teichoic acid is interdependent in pneumococci since the synthesis of PG, which is the basis of cell growth, was inhibited by choline deprivation [116]. The carrier lipid intermediate may be shared by precursors of peptidoglycan and teichoic acid units, i.e TA may be also synthesized in linkage to the carrier lipid, and it is likely that only cholinated TA is transferred across the membrane [97,111]. Thus, teichoic acid units lacking the PCho residue would not be transfer but remain attached to the carrier lipid making this important lipid unavailable for the transport of the muropeptides precursors to the outer

surface of the membrane resulting in the inhibition of cell wall synthesis and bacterial growth [111,117].

The nutritional requirement for choline can be fulfilled *in vitro* by other amino alcohols [118,119] however, choline is absolutely required for normal physiological properties [109,118,120]. *S. pneumoniae* strains growing in medium in which choline has been replaced by the structural analogue ethanolamine show numerous abnormalities including formation of long chains due to block of cell separation at the end of cell division, inhibition of stationary phase lysis (autolysis), inability to undergo genetic transformation and resistance to bacteriophage infection [118]. Phosphorylethanolamine is incorporated into LTA and WTA in place of phosphorylcholine, but it cannot fully replace phosphorylcholine functionally [118,121].

Choline was also shown to be necessary in many stages of pneumococcal virulence [109,122,123]. For example, in a recent study, the absence of choline in the cell wall was demonstrated to inhibit the bacterial capacity to adhere to human nasopharyngeal cells and to completely block invasion [123]. Furthermore, the bacterium was rapidly eliminated from its *in vivo* habitat (nasal passages of mice) and its virulence in both the intraperitoneal and intravenous models was dramatically reduced [123]. The relevant properties for virulence were definitely associated with choline as exposure to this molecule resulted in rapid reversion *in vivo* and *in vitro* to the choline-dependent phenotype [123].

The demonstrated role of choline in pneumococcal physiology and virulence can mainly be attributed to choline functioning as a specific attachment site in the cell wall for a versatile family of noncovalently bound choline binding proteins (CBPs) (Fig.I.8 and I.13) [20,124] that, besides their contribution to virulence (section 5.2) play diverse and crucial roles in bacterial physiology including the cell wall hydrolases (see below a more detailed description of cell wall hydrolases biological roles and importance in pathogenesis). Also, the trimethylamino group of choline residues carry a net positive charge at physiological pH and the absence of these molecules may cause profound and multiple biochemical and structural disturbances at the pneumococcal surface. Choline also interacts directly with the platelet activating factor receptors of host cells promoting pneumococci adherence [125].

3.2. BACTERIAL CELL WALL HYDROLASES

For bacterial cell growth, the cell wall must be continuously restructured. PG synthesis requires degrading preexistent cell wall for the attachment of new constituent molecules, without compromising its structural integrity [105]. This requires the action of cell wall

hydrolases (CWHs), which are enzymes that specifically cleave covalent bonds of the cell wall. When this unregulated hydrolyzing activity weakens the cell wall making the cell unstable and sensitive to osmotic pressure, these enzymes eventually cause the lysis and death of the bacteria and, as such, are designated lysins, autolysins or lytic enzymes [20,105]. Consequently, the term autolysin may not include all cell wall hydrolases.

These enzymes show substrate specificity, related to their interaction with the insoluble cell wall substrate, and bond specificity, which determines the site of action. Based on the bond specificity, that is, the type of bond hydrolyzed, CWHs are classified as: (i) glycosidases, including β-*N*-acetylmuramidases (or lysozymes) and β-*N*-acetylglucosaminidases, which hydrolyze the β-1,4 glycosidic bonds between GlcNAc and MurNAc residues of the glycan strains (lysozymes, between MurNAc and GlcNAc and glucosaminidases, between GlcNAc and MurNAc), and transglycosylases that cleave the same bond that lysozymes but by a different reaction mechanism (ii) amidases, that cleave the amide bond between the MurNAc on the glycan strands and the first amino acid L–alanine of the peptides of the cell wall and (iii) endopeptidases, that hydrolyze with different specificities within the peptide chains of the peptidoglycan [20].

The bacterial CWHs identified and ubiquitously present in *S. pneumoniae* include an amidase LytA, *N*-acetylmuramoyl-L-alanine amidase [98,126,127], and two glycosidases, LytC, a β-*N*-acetylmuramidase (lysozyme) [128], and LytB, a β-*N*-acetylglucosaminidase (Fig.I.10) [129,130]. In addition, phosphorylcholine esterase Pce (or CbpE), which does not target the peptidoglycan but removes phoshorylcholine residues from TAs remodelling the distribution of these residues, is also a pneumococcal CWH (Fig.I.10) [131-133]. Moreover, an endopetidase function seems to be associated with the recently identified cell wall hydrolase CbpD [134].

Besides their role in synthesis and repair of the cell wall, bacterial CWHs, due to their hydrolyzing activity, participate in a variety of fundamental biological functions. LytA is encoded by the *lytA* gene and it is a 318 amino acid protein with a predicted molecular mass of 36.5 kDa [20]. Early studies focusing on its function reported a contribution to the separation of daughter cells at the end of cell division [135-137]. In the absence of LytA activity, pneumococcus grows normally but forms small chains (6 to 8 cells in length) [136]. However, LytA's key function is being the major pneumococcal autolysin [129,135,136], representing the first example of a bacterial autolytic gene that was cloned and expressed heterologously [138,139]. As a potent lytic enzyme [129], it has significant roles in several processes such as self-induced lysis (autolysis) in the stationary phase of growth [135-137,140], competence development during genetic transformation [141,142] and in the irreversible bacteriolytic effects of β-lactam antibiotics [135-137,140,143]. It should be noted that the property of bile

or deoxycholate (DOC) solubility, used to differentiate pneumococci from other streptococci, is a characteristic attributed to triggering of LytA [98,135,140].

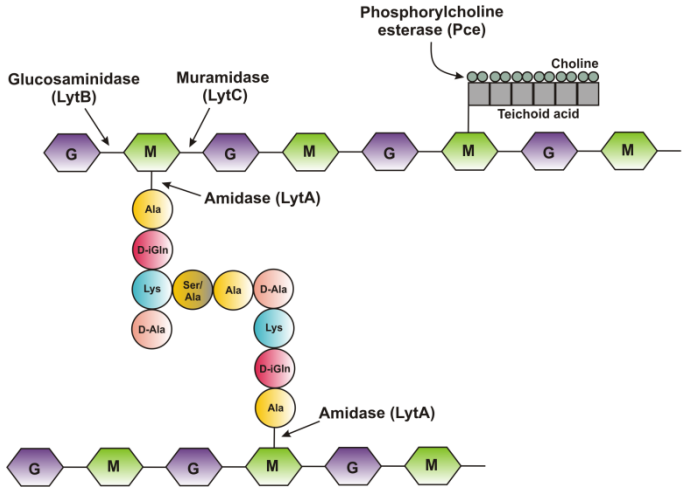


Figure I.10. Schematic representation of the pneumococcal cell wall and the bonds cleaved by the different CWHs LytA, LytB, LytC and Pce. Only two layers of the PG are represented. The bond between peptides (in this case tetrapeptides) is symbolized by two amino acids. G, *N*-acetylglucosamine; M, *N*-acetylmuramic acid.

Interestingly, a mutant lacking *lytA* (M31) displays another autolytic activity in the stationary phase which has been ascribed to LytC [128,129]. This enzyme shows higher activity when incubated at 30ºC but still lower compared to that of LytA at 37ºC (LytA's optimum temperature) [128,129]. A minor lytic function in the response to β-lactam antibiotics might also be attributed to the lysozyme [143].

Although LytB has glucosaminidase activity, its *in vitro* degradation rate of choline-containing cell walls is rather low, in remarkable contrast to LytA and also very distinct from LytC [129]. Consequently, it is considered a nonautolytic hydrolase of pneumococcus. This was unequivocally demonstrated when inactivation of the *lytB* gene did not affect total cell wall hydrolytic activity in the stationary phase of growth and, most importantly, no lysis was observed at either 30ºC or 37ºC in the absence of both LytC and LytA activities [129]. Nevertheless, LytB has a central role in the physical separation of daughter cells, the final event of the cell division cycle [129,130]. Indeed, inactivation of the *lytB* gene in M31 led to the formation of long chains with more than 100 cells [129,130]. This enzyme was found to be localized at the cell poles on the pneumococcal surface which might be indicative of this

critical role [129]. Also concordant, transcription of *lytB*, similar to *lytA*, is high during the exponential phase of growth and decays as the culture enters the stationary phase [20].

3.2.1. AUTOLYSIN LytA CELLULAR LOCALIZATION

As mentioned before, all pneumococcal cell wall hydrolases are CBPs [124,144], therefore located at the cell surface [128,129,132,145] and dependent on choline binding for activity. Indeed, in fractionation assays, LytA was detected in the cell walls [127] and also appears associated with the membrane [146,147]. The surface exposed protein accounts for the majority of LytA in cells although, an appreciable amount occurs in the cytoplasmic fraction [146-148]. This is consistent with the reported interaction with both WTA [121,149] and cell membrane-linked LTA [146]. It should be noted that in intact cells, though LTAs are attached to the cell membrane, they are not entirely physically separated from the peptidoglycan as they can branch into it and therefore become embedded within the structure. Thus, LytA is considered to be cell wall localized.

Accordingly, when ethanolamine was used instead of choline in the growth medium, LytA was totally cytoplasmic [146]. Additionally, as a cell surface exposed protein and due to its noncovalent binding to choline, LytA can be released from the cell surface by 2% choline [146,149,150]. Even though in a few studies the effectiveness of this process was questioned [151,152], it was also demonstrated in parallel assays that the efficiency of removal of another CBP by the same method was dependent on the strain, varying considerable between different capsular types [151]. More importantly, LytA has been located in an external position, predominantly target to the equatorial growing regions of pneumococci (septum at the midcell) being present also at the cell poles (Fig.I.11A) [145]. It was also bound to the outer periplasmic face of the inner cytoplasmic membrane when synthesized in *E. coli* (Fig.I.11B) [90,145]. It can be inferred that in both homologous and heterologous systems, the activity of the enzyme must be regulated, although the nature of the control system in *E. coli* remains fully unknown. In *S. pneumoniae*, the activity of the enzyme is controlled even in two extreme situations such as the hyperproduction of LytA (from inside) [137] or by the addition of the enzyme to the culture medium (from outside) [140] however, in both cases, culture lysis takes place shortly after cells enter the stationary phase in contrast with the normal lysis observed late in stationary phase.

In order to reach the peptidoglycan, LytA must be translocated to the outside of the cell. Surface proteins of Gram-positive bacteria, including cell wall hydrolases are usually targeted for secretion from the bacterial cytoplasm by N-terminal signal sequences [60,153,154]. The

signal sequence allows export across the cytoplasmic membrane by the general secretion Sec pathway, a ubiquitous and essential transport system in every bacterium [155]. This system is well characterized in *E. coli*, consisting basically of a membrane-spanning translocase channel SecYEG (composed of the membrane integral proteins SecY, SecE and SecG) and SecA, that binds to cytoplasmic precursor proteins destined for export and delivers them to the translocase (through its ability to bind both membrane phospholipids and the translocase itself) and is also an ATPase that provides energy for translocation. Through repeated cycles of ATP binding and hydrolysis, SecA undergoes conformational changes that drive stepwise export of a precursor protein through the translocase and across the membrane. During or immediately after transport, the signal sequence is cleaved off from the precursor by a signal peptidase and the resulting mature exported protein then folds into its final conformation [156,157]. It was very recently described that some Gram-positive bacteria, including *Streptococcus* species, have additionally an accessory Sec pathway with an accessory SecA protein called SecA2 [154,158-160]. Like SecA, this protein also has an ATPase activity but is generally not essential for cell viability [156,158,161,162]. Besides the systems that seem to only encompass SecA2, others also contain an accessory SecY2 like in *Streptococcus gordonii*, *Streptococcus parasanguinis* and *Streptococcus agalactiae* [156,160,161,163]. This streptococcal SecA2/SecY2 system includes as well at least three accessory secretion proteins [156,160]. Interestingly, the *secA2* locus in *S. pneumoniae* exhibits the same conserved genes in a similar genomic organization to that found in other streptococci [160,164].

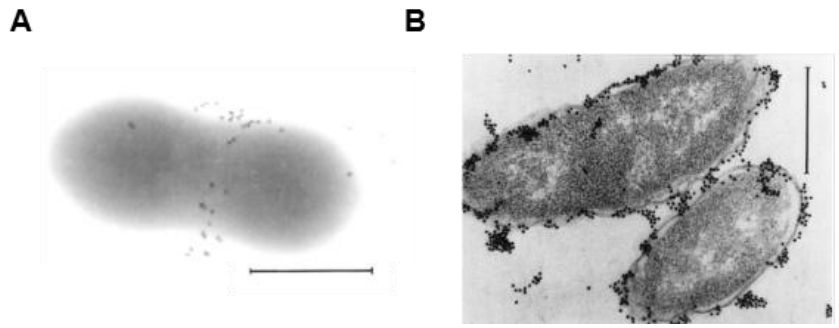


Figure I.11. Immunochemical localization of the pneumococcal amidase in intact cells of *S. pneumoniae* and in ultrathin sections of *E. coli*. *S. pneumoniae* strain M51 grown in medium containing choline (**A**) or ultrathin sections of *E. coli* (**B**) were labelled with anti-amidase serum. Bars represent 0.5 µm. From [145].

In remarkable contrast to all other CWHs (LytB, LytC and Pce) identified in pneumococcus [20,128,129] and the large majority of CBPs [124], LytA does not have a signal peptide (Fig.I.12) [145]. Other sequence signals, besides the canonical signal peptide, or motifs associated with a

surface localization, including the cell wall anchoring motif LPXTG [165], were not found. In *Staphylococcus aureus* a holin-like protein that increases CWH activity was suggested to also assist their transport [166] however, such a protein has not been found in *S. pneumoniae*. Additionally, a zinc metalloprotease, ZmpB, was proposed to control translocation of LytA to the surface possibly by proteolytic release of LytA from the intracellular complex with protein CinA [167]. Nevertheless, this assumption was not reproducible by an independent study. Strain contamination was suggested since the mutant strain used before was not identified as *S. pneumoniae* and differed from its parent strain in several aspects [168]. Consequently, the mechanism of transport of LytA through the membrane to reach the peptidoglycan substrate remains an open question.

3.3. LYTIC ENZYMES PHAGE-BACTERIAL INTERRELATIONSHIP

3.3.1. DOMAIN ORGANIZATION

The lysins that phages encode to release their progeny from infected cells through degradation of the bacterial peptidoglycan are also cell wall hydrolyzing enzymes [56]. Two different classes of lytic enzymes have been found in pneumococcal phages. All of the reported lysogenic phages (HB-3, MM1, EJ-1, VO1 and SV1) as well as the lytic phages Dp-1 and ω-2 possess amidases [24,25,36,169-171], whereas phages belonging to the Cp family encode lysozymes [24,172,173].

The phage lytic enzymes found so far in the pneumococcal system are strikingly related to the lytic enzyme LytA of the bacterium [18,20]. All these enzymes can only hydrolyze cell walls that contain choline in their teichoic acids, with the unique known exception of the lysozyme Cpl-7 from the pneumococcal phage Cp-7 [18,98,121,149,169,173]. The first experimental evidence showing that a pneumococcal phage lysin depended on the presence of choline for activity (*in vivo* and *in vitro*) came from the characterization of the Cpl-1 lysin encoded by the phage Cp-1 [172]. Comparative analysis between this phage lysozyme and the host amidase LytA revealed extensive sequence similarities in the C-terminal regions of both proteins, whereas the respective N-terminal regions were completely different [174]. As both enzymes shared the choline absolute requirement for activity [118,149,172], it was suggested that the pneumococcal autolysin and the lytic enzymes of its phages have two independently functional domains, the C-terminal domain (choline binding domain, CBD) involved in the specific binding to the choline residues contained in the cell wall of pneumococci (i.e. substrate recognition), whereas the N-terminal conferring the specificity for the catalytic activity (Fig.I.12) [174,175].

Further experimental support for this hypothesis was achieved with additional research on several new phage lytic enzymes [18,169,173,176]. The remarkably high nucleotide identity among these phage enzymes and to the host LytA reflected the common features shared between them, with the maximum value of identity of 87% when comparing *lytA* and *hbl* from HB-3 temperate phage (and 90% between amino acid sequences), both encoding a choline-dependent amidase activity [169,176]. Based on this nucleotide identity, *lytA* was and can still be used as a probe in hybridization experiments to detect lytic genes in phages [15,169]. In addition, the lysozyme encoded by the pneumococcal phage Cp-7 contains an N-terminal domain similar to that of the Cpl-1 lysozyme but a completely different C-terminal domain from that of the rest of the enzymes (Fig.I.12) [173]. As a result this enzyme was shown to maintain the lysozyme activity but, as mentioned before, does not require binding to choline for degrading pneumococcal cell walls [92,173].

Moreover, the construction of functional chimeric lytic enzymes [175,177-180], as well as the independent functional expression of the two domains [177,181,182] allowed to further establish the role of each of these domains. For instance, the C-terminal domain of the pneumococcal amidase when independently cloned and produced in *E. coli* is able to bind choline but is unable to hydrolyze cell walls [177]. Fusion proteins generated using individual functional modules of bacterial and phage origins (chimeras) were able to hydrolyze pneumococcal cell walls with the activity characteristic of their catalytic modules [175,179]. This observation suggested that several module combinations are possible *in vivo* through recombination between phage and bacterial genes since they show high identity [175,179,183]. Indeed, the characterization of Cpl-7 [173] and amidase Pal from phage Dp-1 [171] corroborate this assumption. In the case of Dp-1, the N-terminal region is highly similar to the lysins of *Streptococcus mitis* phage SM1 [184] and *Lactococcus lactis* phage BK5-T [185] whereas the C-terminal region is similar to the pneumococcal choline binding domain (Fig.I.12). Thus, the lysin is a natural chimera of intergeneric origin possibly resulting from recombination of phage genes from these species [171].

Studies focused on the choline interaction revealed that, despite the absence of autolysis by pneumococci grown in ethanolamine [98,118,121] due to the lack of specific ligands from the insoluble cell wall substrate, soluble choline- or ethanolamine-containing muropeptides of pneumococcus were hydrolyzed by LytA at similar rates [186]. Furthermore, cell walls isolated from choline-independent pneumococcal mutants grown in the absence of any aminoalcohol, and lacking either choline or ethanolamine in the TAs, are resistant to degradation by LytA [117,120]. It was also observed a complete and rapid hydrolysis when pneumococcal peptidoglycan lacking TAs was used as substrate [120]. This is consistent with the observation

that cell walls from Gram-negative bacteria such as *E. coli*, that do not contain either choline or TAs, are degraded by LytA when expressed concomitantly with the holin of Cp-1 or EJ-1 [90,91]. Equally, the expression of entire phage lytic cassettes in *E. coli* and *Pseudomonas putida* (also a Gram-negative bacterium) revealed that pneumococcal phage lysins (amidases and lysozymes) are also able to hydrolyze the intact PG network of these bacteria [90,91]. Considering all these studies together, a plausible reason for this ability might be the lower structural complexity of the peptidoglycan from Gram-negative bacteria. Presence of TAs chains may actually block access of the host and phage lysins to the pneumococcal peptidoglycan substrate unless this inhibitory effect is somehow neutralized through an interaction of TA-linked choline residues with choline binding sites at the CBD of the enzymes [120].

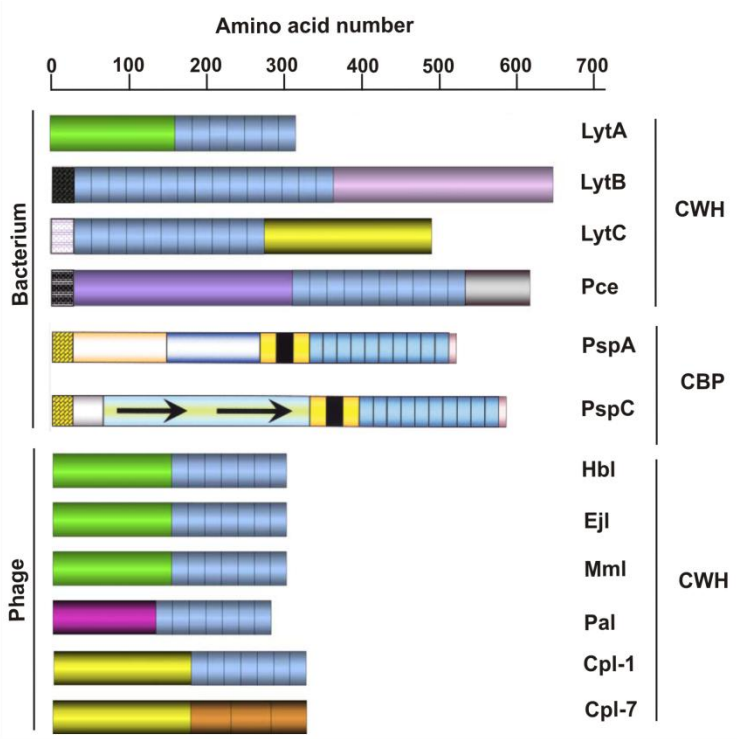


Figure I.12. Diagrammatic representation of the structure of *S. pneumoniae* cell wall hydrolases (host and phage) and of other choline binding proteins. LytA, LytC, LytB and Pce are bacterial CWHs and Hbl, Ejl, Mml, Pal, Cpl-1 and Cpl-7 are phage-encoded CWHs. PspA and PspC or CbpA are also choline binding proteins (CBPs) but without cell wall hydrolyzing activity. The domains containing the active centre of the enzymes are represented for amidase in green or purple (in the case of Pal), lysozyme (yellow), pink (glucosaminidase) or violet (phosphorylcholine esterase, Pce). The choline binding domain (blue) and the choline binding repeats are also shown. The C-terminal of Cpl-7 is coloured in orange. Signal peptides of LytB, LytC and Pce are depicted as small rectangles at the left in the structure. Homology between CWHs is indicated by identical colours. Adapted from [187].

Considering the discussed common properties, lytic genes of phages infecting *S. pneumoniae* were cloned in the homologous system to explore the role of these lysins in bacterial-mediated lytic events [92]. Pneumococcal cultures are prone to lyse under certain physiological conditions without the involvement of a phage-encoded enzyme [135,136], thus a mutant deleted in the *lytA* gene (M31) was used. The expression of either lysozymes (Cpl-1 and Cpl-7) or amidase (Hbl) in this background restored the bacterial wild-type phenotypes [92]. The transformed bacteria underwent extensive lysis in the stationary phase of growth or after deoxycholate addition, events ascribed to LytA [135,136,188], and grew as normal diplococci instead of forming short chains [92]. Although LytC autolytic activity was not inhibited, it contributes little to lysis under those experimental conditions [128,129], so the extensive lytic patterns observed could be essentially attributed to the phage enzymes [92]. On the other hand, expressing in *E. coli* the phage Cp-1 holin concomitant with LytA amidase, instead of the respective phage lysin, resulted in extensive cell lysis [91]. It then became clear that autolysin LytA and *S. pneumoniae* phage lysins could functionally replace one another.

The subsequent characterization of the other choline-dependent pneumococcal cell wall hydrolases LytB, LytC and Pce revealed that these proteins also display a bimodular organization (Fig.I.12). Furthermore, the choline binding domain of the autolysin LytA is, like in phage lysins, constituted by six repeated motifs (known as choline binding repeats, CBRs) of 20-21 amino acids and similar motifs have been detected in other CBPs (Fig.I.12) [20,30,170,173,177,178,189]. However, in LytB and LytC, the CBD was unusually found in the N-terminal region whereas the active site of the enzymes was localized in the C-terminal domain (Fig.I.12) [128,129]. Also, the number of CBRs varies among the CBPs with 10, 11 and 15 present in Pce, LytC and LytB, respectively (Fig.I.12) [20,128]. Interestingly, LytB is able to bind to ethanolamine-grown cells (although the long chains remain uncut) [129], in sharp contrast with LytA [145,149]. Additionally, a comparison of the lytic enzymes from Gram-positive bacteria and their phages did not indicate such a high degree of structural and functional similarity as that showed between LytA and *S. pneumoniae* phage lytic enzymes. Still, the modular organization with separate catalytic and cell wall binding domains (targeting proteins to the bacterial surface) is a general characteristic of Gram-positive bacteria cell wall hydrolases (phage-encoded and bacterial), in which the catalytic activity is almost always located in the N-terminal region while the C-terminal part contains the substrate recognition and binding domain [190-193]. Indeed, a relationship was reported, based solely on amino acid sequence identity, between three different lysins from *Bacillus cereus* phages, showing remarkable heterogeneity among them, and different autolysins but from other bacilli [190].

3.3.2. REGULATORY MECHANISMS

Besides the strict requirement for choline in the teichoic acids to degrade the pneumococcal cell wall [98,149,173], since specific attachment to choline localizes the lysin catalytic domain near the peptidoglycan substrate, another interesting property shared between pneumococcal phage-encoded enzymes and the host LytA is the intimate involvement of choline of TAs in the regulation of the lytic activity at the post-translational level [121,126,146,169,170,194,195].

It is now known that the interaction with choline at the cell surface is crucial for autolysin activation [121,126,146,148,189]. In early studies, it was reported the puzzling observation that ethanolamine grown pneumococci not only contain LytA resistant cell walls but, in addition, the normal autolytic enzyme characteristic of choline-grown bacteria (with high catalytic activity, later named the C-form autolysin) is missing from such cells and is replaced by one with a low catalytic activity (E-form autolysin), suggesting a more important function for choline than just a binding ligand [121,148]. Indeed, choline (TA-bound) could mediate the "conversion" *in vivo* and *in vitro* of the low active E-form of LytA into the fully catalytically active C-form [121,126,146,148]. In addition, the low active E-form of the enzyme was detected only in the cytoplasmic fractions of choline-grown pneumococci whereas the prevalent C-form was present at the cell surface, since only at the surface are possible the interactions between the autolysin and TAs [146,148]. Concordantly, the single E-form present in cells grown with ethanolamine was found only in the cytoplasm when investigated in different cellular fractions [146]. These findings have been further corroborated by the elucidation of the biochemical role of CBRs in conversion, revealing that LytA depends on the binding of a minimum of 4 CBRs repeats on the C-terminal domain for enzymatic activity and the catalytic efficiency increases directly with the number of repeats involved in the choline interaction [30].

Likewise, choline binding also seems to induce and stabilize the active conformation of all the phage lysins of *S. pneumoniae* studied so far. For instance, the phage lysin encoded by phage HB-3 can be converted to a more active form under conditions similar to those described for the pneumococcal autolysin [148,169]. Structural and thermodynamic characterization of Pal of phage Dp-1 revealed that choline interaction strongly stabilizes the cell wall binding module, and the conformational stabilization is transmitted to the catalytic region. These structural rearrangements triggered by choline may underlie the *in vitro* "conversion" of Pal from the low to the full activity form [196]. The specific recognition of choline by the lysin of phage Cp-1 was also demonstrated to enhance its catalytic activity and

the stability of the enzyme [181,195]. Finally, choline induces alterations in the Ejl encoded by phage EJ-1 [170] resembling those induced in the bacterial LytA, which have been related to increased catalytic efficiency [189].

Apart from the converting (activating) capability of the LTA [146], it was also proposed that the activity of LytA could be inhibited by the choline-containing lipoteichoic acid [126,146,194]. However, the inhibitory role of LTA and the mechanism underlining it are unclear. On one hand, Höltje and Tomasz reported that LytA activation is mediated only by the pneumococcal choline-containing cell wall teichoic acid because, under the experimental conditions used, LTA inhibits the autolysin activity in contrast to WTA [126,194]. On the other hand, subsequent studies argued that the LTA inhibitory effect reported previously was due to the formation of micelles by LTA in aqueous solution (as LTA contain lipid moieties) preventing the access to the substrate of the bound autolysin, since LTA stripped of its lipid moiety lost its capacity to inhibit the autolysin to the same extent that it lost its capacity to form the "micelle traps" [146]. Still, it was not discarded the possibility that *in vivo* the LTA could constrain autolysin activity. It was suggested that LTA might constitute a topological barrier, similar to the *in vitro* situation when the autolysin is bound to LTA-micelles and is thereby hindered from reaching the peptidoglycan substrate [146].

In similar studies, free choline was shown to interfere with the interaction between choline-containing teichoic acids and LytA [127,146,149]. Indeed, high choline concentrations (2%) completely inhibit the enzyme *in vitro* [146,149]. Even *in vivo*, 2% choline in the culture medium leads to phenotypically autolysin-deficient pneumococci [146]. This inhibition was attributed to desorption of the enzyme from its substrate by choline [149]. Most likely, it occupies the same sites that allow the enzyme to recognize and interact with the teichoic acid-bound choline [149], explaining the observed release by choline of LytA associated with both LTA and WTA [146,149]. Similarly, other choline binding proteins are also dissociated by choline [151].

Curiously, free choline and lipoteichoic acid also inhibited the activity of the Cp-1 phage lysin Cpl-1 [172], tested *in vitro* essentially according to the method of Höltje and Tomasz [194]. Therefore, the activity of Cpl-1 might be hindered by LTA as proposed for LytA, although the physiological relevance of this putative regulation still remains an open question since pneumococcal phage lysins are generally believed to be cytoplasmic (thus, their activity would be physically hampered until holins set the time of lysis).

Since the autolytic LytA enzyme plays important roles in complex physiological processes (such as cell wall enlargement and cell division) and its cell wall hydrolyzing activity is potentially lethal to the bacterial cell, it is understandable that a variety of control strategies

exist. Other than the direct interactions with TA-choline, the teichoic acid phosphorylcholine esterase could offer an additional potential regulatory mechanism through the removal of a critical set of phosphorylcholine residues from either the cell wall teichoic acid or from the lipoteichoic acid, which is also a substrate for the esterase [131]. The selective removal of a fraction of choline residues by this enzyme is likely to create autolysin resistant regions in the cell wall. In the same way, the potential inhibitory influence of lipoteichoic acid may be affected by the removal of phosphorylcholine residues [194]. As far as the expression of the *lytA* gene is concerned, characterization of the transcript of the autolysin did not reveal the presence of putative transcriptional or translation regulatory structures [197]. Although *lytA* is constitutively expressed during the exponential phase of growth [197,198], more recently a distinguishable transcriptional regulation of *lytA* was described during competence development with higher expression of the autolysin. *lytA* is located in a competence-induced operon being cotranscribed under competence conditions with *recA* [198-200].

Other levels of LytA regulation at the cell surface may include the energy status of the cytoplasmic membrane [90], paralleling what happens in other Gram-positive bacteria. It was described that the bacteriocin-induced lysis of some *Lactococcus* and *Lactobacillus* strains is a consequence of bacteriocin-induced depletion of cellular energy [201]. Moreover, it is known that pmf-dissipating agents are able to trigger lysis in *Bacillus subtilis* [202,203]. It is worth noting that, despite two main bacterial lysins (the amidase LytC and the putative endopeptidase LytE) having been implicated in cell lysis triggered by energy poisons, the major *B. subtilis* glucosaminidase LytD, also a secreted enzyme, appears not to participate in this response [204,205]. This set of data therefore suggests that whereas some enzymes respond to pmf-dissipating factors, others have their activity stimulated in different ways, still unknown, even within the same species. Whether the control of the pneumococcal autolysin activity depends on an energized membrane remains to be determined.

4. LYSIS IN *S. PNEUMONIAE*

Besides phage-mediated host lysis, *S. pneumoniae* also undergoes lysis mediated by its own autolysin LytA under several physiological circumstances [135,136,141], as mentioned earlier. For instance, it has been consistently observed the self-induced lysis (autolysis) *in vitro* in the stationary phase of growth [135-137,140]**.** Direct experimental evidence for the essential role of LytA in this autolytic event was provided by studies with autolysin defective pneumococci [135,136], where restoring the enzyme reverted the cells to the wild-type phenotype of stationary phase lysis [137,140]. Although autolysis has also been reported for

other bacterial species, the extensive lysis caused by LytA in this stage is a unique feature of *S. pneumoniae*. However, how and why autolysin control is relieved remains unknown. It was suggested that it could be an advantage for pneumococcal survival under prolonged periods of nutritional deprivation, serving the contents of the lysed cell as a nutrient source, since in a fully lytic culture it was observed that some viable cells remain for a considerable amount of time while in a *lytA* mutant derivative of the same strain there were no long-term survivors, even though there was no culture lysis in the stationary phase [206].

Bacterial lysis mediated by LytA has also been implicated in competence to undergo genetic transformation [141,142]. Competence, the ability of cells to take up free DNA from the surrounding medium, is a transitory property in *S. pneumoniae* which develops suddenly during the early exponential phase of growth [141]. During the development of this process, release of transforming DNA was repeatedly observed [141,207]. It was proposed that the competence-induced liberation of DNA from the cytoplasm to the medium is mediated by LytA-dependent cell lysis (autolysis), since *lytA* mutants have significantly less amount of DNA available in the medium, and that it occurs in a subfraction of the actively growing bacterial population where the majority of cells acts as a recipient of the DNA from the medium [141]. Other more recent studies reported that competence-induced pneumococci lysed competence-deficient cells during cocultivation (allolysis) [142,208,209]. LytA of competent attackers is also important in this process and in noncompetent target cells LytA can as well be *trans*-activated by competent cells [142,152,208]. Even though, allolysis promotes DNA release, it was not ruled out that autolysis could operate simultaneously [142].

Besides cell-regulated processes, it is known that β-lactams trigger pneumococcal LytA-mediated lysis [135,136,140,210]. These compounds inhibit the transpeptidase activity of cell surface localized PBPs and consequently cell wall synthesis in growing cells resulting in arrest of bacterial growth (bacteriostatic response). Additionally, inhibition of PG synthesis also leads to uncontrolled activity of LytA which inevitable ends up in cell lysis (bacteriolytic response) [206,210]. The mechanisms connecting the interaction of β-lactam with its PBP target and LytA activation are not known despite clues about some factors involved exist. For instance, besides cell lysis, cell death is also induced by penicillin independently of LytA involving the putative Cid protein and this protein was implicated in triggering LytA activity [211]. A model for *S. pneumoniae* penicillin induced-lysis via Cid was speculated resembling the phage classical holin-endolysin mechanism of host lysis assumed for pneumococcal phages. Normally, the Cid protein would operate at the cytoplasmic membrane providing a mechanism of controlled transport of LytA to the pneumococcal cell wall, which guarantees negative regulation of its activity at the cell surface. When performing this physiological function, Cid would exist in low

numbers per cell being the *cid* gene under precise control. However, a substantial antibiotic-induced increase in the number of copies of this protein would be toxic for the bacterium. At a higher cell concentration, Cid would assume a holin-like function injuring the cytoplasmic membrane, probably by increased permeability, causing not only cell death due to loss of membrane integrity but allowing the release from the cytoplasm to the cell wall of large amounts or unregulated forms or even both of the autolytic enzyme [211]. However, the gene(s) responsible for the *cid* phenotype were not yet identified. Curiously, in subsequent studies it was demonstrated that the CidA protein of *S. aureus* is actually a phage holin-like protein that controls cell death and lysis forming membrane lesions [212]. Accordingly, CidA has a positive effect on cell wall hydrolase activity and results in an increased sensitivity to penicillin-induced killing [166]. Still, the mechanisms through which this membrane disturbance triggers the activity of bacterial cell wall hydrolases activity are unknown.

Notably, bacterial lysis is associated with the *S. pneumoniae* pathogenic potential. Thus, its role, including in the context of biofilms, will be further discussed.

5. LYSIS AND PNEUMOCOCCAL VIRULENCE

5.1. PNEUMOCOCCAL VIRULENCE

S. pneumoniae is usually found as a commensal organism in the human host colonizing asymptomatically the nasopharynx of healthy individuals [3,213,214]. Although multiple pneumococcal serotypes can be carried in the nasopharynx, some of the most common serotypes isolated from carriage are also those serotypes most commonly associated with invasive disease [2]. This, together with the observation that disease most likely occurs by a serotype that recently colonised the host [215] strongly suggests that carriage is the first step in the ability of the pneumococcus to cause infection. For dissemination from the nasopharynx to other parts of the host (e.g. middle ear, lung, brain), *S. pneumoniae* must cross tissue barriers and be able to adapt to different environments within the host. Despite the precise mechanisms being unclear, this transition into invasive pathogen is a complex process that involves several bacterial virulence determinants. Also, host factors clearly play a role in the susceptibility to this bacterium [2,213,216].

5.2. IMPORTANT VIRULENCE FACTORS

5.2.1. POLYSACCHARIDE CAPSULE

The polysaccharide capsule, that covers the cell surface of the pneumococcus, is considered one of the most important factors in its ability to cause infection [20,217,218]. In fact, nonencapsulated strains are rarely isolated from patients and have greatly reduced virulence (being considered mostly avirulent) compared to the respective encapsulated counterparts [3,217,219]. The capsule is important during invasive infection, presumably because it confers protection to the bacterium from phagocytosis by blocking the opsonization directed against cell surface antigens [20,217,218,220]. Despite the diversity of the capsular polysaccharides (more than 90 different capsular serotypes are known so far [6-9]), all perform this same primary function even though different capsular types differ in their resistance to phagocytosis [220]. As a highly immunogenic material, it elicits a large immune response and as a consequence of compromised phagocytic clearance, the increase in these responses causes even more tissue damage at the infection site, which may facilitate further invasion [218].

Furthermore, studies have shown that the capsule is not only important in disease but for colonization as well [221,222]. Due to the presence of a capsule, pneumococcus is able to escape from entrapment in the mucus thus, is more likely to transit to the epithelial surface where stable colonization occurs [221]. It also appears that the capsule interferes with the formation of biofilms, the preferred growth modality of *S. pneumoniae* during colonization [223].

5.2.2. SURFACE PROTEINS

The bacterial surface also plays a major role during pneumococcal disease, as it represents the interface on which the cell interacts with the host. Many pneumococcal virulence factors are indeed located in the cell surface including choline binding proteins (Fig.I.13). For example, choline binding protein A, CbpA (also known as SpsA, *Streptococcus pneumoniae* secretory IgA binding protein, and PspC, pneumococcal surface protein C), the most abundant choline binding protein [224], has been shown to be involved in adherence to endothelial cells and lung epithelium [224,225], which is probably related to its observed role in colonization [224,226,227] and invasion of the lung tissue *in vivo* [226,228].

Likewise, mutants lacking PspA (pneumococcal surface protein A), another cell surface exposed CBP, are attenuated in animal models [229]. In invasive infections, it was proposed

that the protein exerts its virulence function by interfering with complement-dependent host defense mechanisms. By inhibiting complement deposition and activation on the bacterial surface, it reduces the effectiveness of clearance [230,231]. On the other hand, PspA might act by a very different mechanism in mucosal carriage, where complement concentrations are especially low [232]. It has been suggested that *S. pneumoniae* may use PspA to overcome the iron limitation at mucosal surfaces, since it binds human apolactoferrin (the metal-depleted form of lactoferrin) [233,234], an iron chelating protein which can deplete this metal and restrict bacterial growth protecting the host from bacterial infections by prevention of colonization [235]. Besides its bacteriostatic activity, apolactoferrin is also bactericidal against *S. pneumoniae* [236,237]. Interestingly, PspA binding to apolactoferrin was found to offer as well significant protection against pneumococcal killing [237].

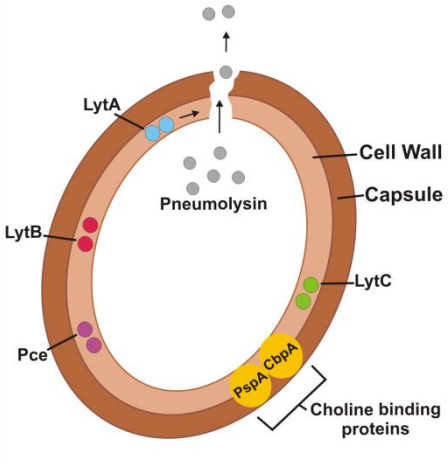


Figure I.13. Some of the major pneumococcal virulence factors. Those include the polysaccharide capsule, pneumococcal surface proteins including choline binding proteins such as LytA, cell wall fragments and pneumolysin (Ply). The CBPs LytB and LytC are also represented even though their roles in pathogenesis are not yet fully understood. Lysis by LytA releases large amounts of Ply.

5.2.3. PNEUMOLYSIN

The pneumolysin (Ply) is the major pneumococcal toxin functioning outside the bacterial cell [3]. It is present in almost all isolates that cause infection [238]. This toxin is believed to have multiple functions in virulence as it possesses both cytotoxic and proinflammatory properties [3]. Pneumolysin binds to cholesterol in host cell membranes inducing lysis and apoptosis by forming transmembrane pores [3,217,238]. This allows the access of the pneumococcus to tissues where it proliferates and initiates inflammatory responses [238]. For instance, by disrupting the blood-brain barrier, this toxin may play a crucial role in bacteraemia. In fact, absence of Ply function delayed bacteraemia in animal models [239]. At sublytic concentrations, Ply also has several effects namely it stimulates the production of inflammatory cytokines such as tumour necrosis factor-α and interleukin-1β [240], decreases

the bactericidal activity and migration of neutrophils [241] and activates the classical complement pathway [242] thereby causing inflammation tissue damage.

5.2.4. CELL WALL HYDROLASES

The cell wall hydrolases LytB, LytC and Pce (Fig.I.13) were recently associated to pneumococcal pathogenesis although their roles are still poorly understood [130,132,243]. Despite these proteins having no effect on virulence in a model for pneumococcus-induced sepsis, loss of function of these proteins in rat models reduced significantly colonization of the nasopharynx [243]. For LytC and Pce, this can be attributable to the observed reduced adherence to nasopharyngeal cells at 30ºC [243] since the temperature in the nasopharynx is usually lower than the normal body temperature of 37ºC. Interestingly, LytC is a minor lysin *in vitro* with higher activity at 30ºC [128] and this is also consistent with a potential role on colonization. On the other hand, LytB is fundamental for pneumococcal cell separation [129,130] and it has been proposed that bacterial chain formation limits the dissemination of the bacteria during colonization and infection and the increased target size of pneumococcal chains may increase the efficiency of phagocytosis in eliminating the bacteria [20,123,130].

5.3. BACTERIAL LYSIS

Lysis by LytA of pneumococci has been strongly implicated in pneumococcal pathogenesis [226,239,244,245]. Indeed, *S. pneumoniae* strains without functional LytA showed *in vivo* reduced virulence relative to that of the wild-type strain [244,245] and mice immunized with purified autolysin survived significantly longer than nonimmunized mice after exposure to a *S. pneumoniae* virulent strain [244]. Additionally, it has been shown that the absence of LytA alters the course of pneumococcal disease [239], but it were also reported minor differences in mice survival time upon infection with pneumococcal strains lacking LytA activity relative to the wild-type strains [135].

One of the direct implications is the release upon lysis of cell wall components, including peptidoglycan fragments and teichoic acids, that were demonstrated to elicit strong inflammatory responses during infection [3,246-248]. Lysis-induced inflammation seems to contribute to tissue injury [249], which may thereby increase permeability of host barriers and contribute significantly to the severity of the infection. In fact, in an animal model of *S. pneumoniae* meningitis, treatment with a nonbacteriolytic antibiotic resulted in the release of smaller amounts of teichoic acids [250] and in a decrease of mortality [251].

It has also been suggested that LytA-induced lysis influences pneumococcal pathogenesis by releasing intracellular virulence factors [3]. I*n vitro*, lysis induces the release of pneumolysin in the stationary phase and during competence development (Fig.I.13) [208,244,252]. A functional link between lysis and pneumolysin was also established *in vivo* based on the observations that mutants deficient in production of both Ply and LytA were no less virulent than strains carrying either Ply or LytA alone [245], and the level of protection in mice immunized with autolysin or pneumolysin were shown to be similar with no detectable additional protection occurring in animals immunized with both proteins [253]. However, the interplay between LytA and pneumolysin is controversial. Even though it was thought that the extracellular presence of Ply was only due to autolysin-induced lysis, significant amounts of Ply were observed to be released from pneumococci during log-phase growth when lysis is not evident [252]. The lysis requirement for this process was questioned when the pattern of Ply release during exponential phase in the absence of LytA or in the presence of 2% choline (that suppresses lysis) was shown to be similar to that of the wild-type strain or in growth in normal choline concentrations [252]. Accordingly, under some infection conditions, mutations in pneumolysin and autolysin had different effects on virulence [252]. In addition, in a subsequent study it was shown a cell wall localization for Ply [254]. Nevertheless, although the exclusive lysis-dependent release of Ply has been disbelieved [252,254], in those studies when cell lysis does occur a large quantity of Ply is made available extracelullarly in culture supernatants [252,254]. In agreement, reduced release of Ply *in vivo* and *in vitro* was detected upon the use of nonbacteriolytic antibiotics in comparison to a bacteriolytic antibiotic [255].

More recently, it was proposed that bacterial fragments generated during autolysis prevent the phagocyte-mediated elimination of live pneumococci, a property frequently associated with the pneumococcal capsule [256]. Although less studied, LytA-induced lysis could also influence virulence by providing DNA for genetic exchange. Indeed, *in vitro* DNA release was observed both in competence and in stationary phase [207,208,257]. It is possible that through this process bacteria could acquire properties more favorable to persistence in the host during colonization or that play a role in infection or in antibiotic resistance [257,258].

5.4. LYSIS IN PNEUMOCOCCAL BIOFILMS

5.4.1. PNEUMOCOCCAL BIOFILMS

In most environments, bacteria form surface-associated organized communities of aggregated cells embedded in an extracellular polymeric substance (EPS) matrix, called biofilms [259-261]. These complex structures are known to comprise one species or multiple

species existing in a mixed community [262,263]. Biofilm growth starts when single planktonic cells attach to a surface that normally is conditioned to enhance attachment. In early formation, those individual cells strongly adhere and proliferate into dense cell groups and, as this occurs, they become surrounded by the extracellular matrix. During subsequent maturation, parts of the biofilm can disperse and migrate. In addition, the biofilm can continue to develop into a heterogeneous population of cells that are metabolically, physiologically and genetically distinct from one another [263]. Dispersion events can result in bacterial dissemination and colonization of new niches [264].

Due to its remarkable difference from planktonic growth, biofilm development allows bacteria to survive in hostile environments [259,261,265]. In the human host, biofilm formation is clearly an important microbial survival strategy. Since the EPS matrix is difficult to permeate, they are inherently less susceptible to antimicrobial agents, even if the individual cells are highly susceptible, and resist attack and killing by the host immune system [259,266]. Several other factors and mechanisms to evade elimination are intrinsic to this bacterial lifestyle [263]. For instance, some cells in biofilms are able to exist in a metabolically dormant state (growth-limited bacteria) which may confer protection against many drugs that target actively growing bacterial cells [267].

It has been estimated that biofilms are involved in 60% of all human bacterial infections and that they are essential for many infections to occur [259,268]. Indeed, biofilm formation *in vivo* was demonstrated for numerous bacterial pathogens, such as *Pseudomonas aeruginosa* in cystic fibrosis-related lung infections, *Haemophilus influenzae* in chronic otitis media, *Staphylococcus aureus* in chronic rhinosinusitis and *Escherichia coli* in urinary tract infections [259,261]. The presence of these structures at the site of infection associates them with these disorders. Furthermore, in some of the infections, biofilm formation is actually an absolute requirement [259,261].

In *S. pneumoniae,* biofilm formation has also recently been shown *in vitro* and *in vivo* [223,269-274]. In fact, pneumococcal biofilms were demonstrated to be locally present in infected tissues of individuals with chronic rhinosinusitis [272] and chronic otitis media [273]. Moreover, in a chinchilla model of otitis pneumococci were shown to form *in vivo* biofilm communities, further supporting the hypothesis that this type of growth plays an important role in pneumococcal infection [275]. Despite not having found an association between the ability to form biofilms *in vitro* and the capacity to colonize the nasopharynx, cause pneumonia and enter the bloodstream (invasive infections) in a mouse model [276], another study established that in the nasopharyngeal colonization, biofilms are likely to contribute to this process since biofilm defects *in vitro* normally impaired colonization *in vivo* [223]. Additionally,

it was observed that the pattern of pneumococcal gene expression, including virulence factors, in *in vitro* biofilms was highly similar to the one of bacteria in tissue infections (brain and lungs), while the expression pattern in liquid growth resembled that of bacteria infecting the blood [271]. Furthermore, pneumococci grown in a biofilm were more effective in inducing tissue infection (meningitis and pneumonia) differing from cells from liquid culture that were more effective in causing blood infection. These findings reveal that during tissue infection pneumococci are most probable in a biofilm-like state and therefore, strongly suggest that the ability to form biofilms is important in these cases [271]. Despite this, little is still known about the molecular mechanisms governing pneumococcal biofilm development.

Concerning the involvement of proteins in *S. pneumoniae* biofilm formation, in a recent work a proteomic analysis over the course of biofilm growth revealed a differential production of proteins [269]. Also, a remarkable increase in the number of detectable proteins was observed in biofilm relatively to liquid growth and it was suggested that such dramatic changes could be partly explained by transition/adaptation to the complex sessile mode of growth [269]. Protein identification revealed that proteins involved in attachment, bacterial resistance and virulence were more abundant under biofilm growth conditions [269]. This seemed related with the referred upregulation of pneumococcal virulence genes in tissue infection (associated with biofilm growth) [271]. In addition, in a quest for the role of choline binding proteins, mutants in LytA as well as in LytC and LytB, showed a decreased capacity to form biofilms *in vitro*, whereas no such reduction was observed, for instance, in Pce mutants [270]. Nevertheless, the role of the autolysin LytA, and of the other proteins, in establishing robust biofilms remains to be determined.

5.4.2. ROLE OF LYSIS

The EPS matrix, constituting a major part of the biofilm, is typically produced by the bacteria themselves within the biofilm [277]. It is a complex mixture of macromolecules that in general includes polysaccharides, proteins and DNA [263,266]. In *S. pneumoniae*, increasing evidence suggests that extracellular polysaccharides indeed form part of the biofilm matrix, whether the biofilm is formed by strains containing a polysaccharide capsule or by nonencapsulated strains [274,278]. Proteins and extracellular DNA (eDNA) were found as well as integral parts of the pneumococcal biofilm matrix [270,274,278].

The matrix eDNA, initially presumed to be a residual material, is actually essential in the biofilm mode of life serving as a nutrient source [277], allowing horizontal gene transfer between cells (facilitated by the closest proximity within the biofilm) [279], and even

mediating antibiotic resistance [280]. Remarkably, it has become increasingly clear that eDNA is also a critical component of the biofilm structure [281-286]. It is in fact required for biofilm formation in several species including Gram-positive bacteria [281-286]. For example, in *Listeria monocytogenes* and *Bacillus cereus* it was reported that eDNA can promote the initial surface attachment imperative for further biofilm growth [282,286]. Furthermore, in *Staphlylococcus aureus* and *L. monocytogenes,* for instance, eDNA was found to be not only responsible for early biofilm formation but also for cell interconnection maintaining established biofilms, mainly based on the observation that DNase I treatment caused significant dispersal of the preformed structures [284,286,287]. However, in other studies, developed biofilms seem to be far less susceptible to DNase I treatment, as in the case of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, even though DNA is important for initial establishment of the biofilm [281,284,288]. Interestingly, it was observed that eDNA is present as a fibrous network in *Enterococcus faecalis* [283] and a grid-like structure was visible for *P. aeruginosa* [289], which is linked to the structural stability conferred by this compound [281,283].

In *S. pneumoniae*, the presence of eDNA was also found to influence biofilm formation. When pneumococci were grown in the presence of DNase I, the development of this structure was indeed greatly impaired, while culture growth was not affected [270]. Moreover, a significant inhibitory effect on biofilm formation was observed adding DNase I after 6 h of growth [270]. Nevertheless, in another study, it was reported the absence of any effect of DNase I whether before or after biofilm development [223] and these discrepancies are not yet understood. Despite this, an additional independent analysis, corroborated the first findings as it was shown that DNase I treatment significantly reduced biofilm biomass [278], further implicating eDNA in the establishment of pneumococcal biofilms.

Concerning the source of eDNA for the biofilm, it has been suggested for some bacteria that active secretion mechanisms may exist [281,282]. Even though the possibility of such mechanisms was not excluded, growing evidence especially in Gram-positive bacteria, support the hypothesis that eDNA is mainly released from cells through lysis [283,287-293]. In *E. faecalis*, impairments in biofilm formation occurred in the absence of the autolysin Atn due to defects in DNA release [283]. Likewise, in *S. epidermidis* biofilms, most of the eDNA promoting biofilm formation is made available by the autolysin AtlE, probably through lysis of a subpopulation of the bacteria [288]. Similar findings were also shown during biofilm development in *S. aureus* involving the holin-like protein Cid, which as mentioned before influences the autolytic activity in this species [287,291,292], and in *Streptococcus gordonii* the release of eDNA by the major autolysin was suggested to be spatially or temporally regulated

for optimal development of stable biofilms [290]. There might be a parallel system in *S. pneumoniae*. As a matter of fact, the major pneumococcal autolysin LytA was shown to influence biofilm formation [270] and in pneumococcal liquid cultures, it was demonstrated that DNA is released into the medium through LytA-induced lysis, whether in the stationary phase or by a fraction of the bacterial population in competence induction [141,142,207]. Thus, within the biofilm limited lytic events mediated by LytA could promote eDNA release favoring its formation, highlighting the need to test this hypothesis.

5.5. ASSOCIATION BETWEEN PNEUMOCOCCAL PHAGES AND VIRULENCE

Dissemination of genetic information among bacteria is facilitated by phages and it is also well documented that some lysogenic phages bear virulence-related genes [12,294]. Toxins are the best recognized examples of virulence factors encoded by phages in several bacterial pathogens, with even more severe consequences upon prophage induction [12]. However, it has become clear that prophages have a broader contribution to their host virulence. For instance, some phages also encode regulatory factors that increase expression of virulence genes not encoded by the phage [295], while others encode enzymes that alter bacterial components related to virulence [296].

The abundance of lysogeny among *S. pneumoniae* strains associated with human infections [15,25,36,42-44] has raised the possibility that phages may influence bacterial virulence. However, this putative phage participation remains elusive since sequence analysis of the pneumococcal genomes failed to reveal any gene potentially involved in host pathogenesis [20,24]. Even though an improved adherence of pneumococci to pharyngeal cells *in vitro* was reported as specifically associated with the MM1 prophage, it remains unknown whether it provides an advantage in colonization [297].

Autolytic events have been strongly implicated in *S. pneumoniae* virulence mostly by massive release of proinflammatory cell wall fragments and intracellular factors [3,217]. Additionally, although no direct evidence exists, it seems that controlled bacteria-mediated lysis may influence positively the formation of pneumococcal biofilms [270], the importance of which has been increasingly recognized *in vivo* in both colonization and infection [223,272,273]. Since it is now clear that all pneumococcal phages encode their own lysins, prophage induction (occurring in both substantial and spontaneous fashion) may contribute to virulence through lysis. In this context, it is important to study the precise mechanisms involved in phage-mediated lysis as well as the impact of this process on *S. pneumoniae*, especially within biofilms of lysogens where the lysis phenomenon is not yet understood.

It is known that *S. pneumoniae* phages depend on the phage-encoded functions of holin and lysin to accomplish lysis and release of their progeny [20,24,25,36,90,91], where the phage lysin has been shown to hydrolyze the pneumococcal cell wall whereas holin compromises the cytoplasmic membrane integrity [90,91]. On the other hand, the major pneumococcal autolysin LytA is localized inactive in the cell wall [126,127,145,146,194] and, in some Gram-positive bacteria the activity of autolysins is apparently regulated at the bacterial surface through the membrane electrochemical gradient [201-203]. Taking into account the powerful lytic activity of LytA [128,136], the membrane disruption caused by the holins may trigger the host autolysin that could also contribute actively to the release of the newly formed phage particles.

Furthermore, experimental evidence demonstrating the peculiar high structural and functional similarity between pneumococcal autolysin and phage lysins has been gathered [20,92]. This is best exemplified by the constitutive expression of the phage lysins Cpl-1 or Hbl in *S. pneumoniae* M31 (deleted in the *lytA* gene), that reestablished the capacity of the cells to undergo autolysis in stationary phase [92]. It then seems that the phage lysins may be regulated by the same bacterial physiological mechanism that controls LytA activity. Additionally, it appears reasonable to hypothesize an extracytoplasmic targeting of the phage lysins since the cell wall located LytA also lacks an N-terminal secretory signal sequence [145]. Therefore, this invites speculation that the phage lysins might actually be activated by holin-induced membrane deenergization at the end of the lytic cycle, in sharp contrast to the mechanism generally accepted in *S. pneumoniae* of the holin-dependent export of phage lysins to their peptidoglycan target [20,24,91,93]. Overall, a simple strategy for phage release can be envisaged: host lysis mediated by phages might be achieved by holin activation, through collapse of membrane energization, of both phage and bacterial lysins already positioned at the cell wall.

From the host perspective, the eDNA constituent of the biofilm matrix [263,266] has been suggested not only to be required for the biofilm development of several bacteria [281-283], but also to influence this process in *S. pneumoniae* [270,278]. Furthermore, autolysins of some Gram-positive bacteria play a role in biofilm growth, probably by providing a source of eDNA [283,288,293,298] and it was observed that pneumococcal mutants in the autolysin LytA have a decreased capacity for establishing biofilms [270]. Thus, the implication of LytA in the formation of these multicellular structures could be explained by eDNA release upon regulated cell lysis. In this scenario, since lysogenic phages are normally spontaneously induced resulting in cell lysis [35,39-41], it can be hypothesized that prophage of a pneumococcal subpopulation

might also participate in eDNA release within the biofilm and consequently enhance its formation.

6. CHAPTER REFERENCES

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CHAPTER II

THE AUTOLYSIN LytA CONTRIBUTES TO EFFICIENT BACTERIOPHAGE PROGENY RELEASE IN *STREPTOCOCCUS PNEUMONIAE*

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1. SUMMARY

Most bacteriophages (phages) release their progeny through the action of holins that form lesions in the cytoplasmic membrane and lysins that degrade the bacterial peptidoglycan. Although the function of each protein is well established in phages infecting *Streptococcus pneumoniae*, the role - if any - of the powerful bacterial autolysin LytA in virion release is currently unknown. In this study, deletions of the bacterial and phage lysins were done in lysogenic *S. pneumoniae* strains, allowing the evaluation of the contribution of each lytic enzyme to phage release through the monitoring of bacterial-culture lysis and phage plaque assays. In addition, we assessed membrane integrity during phage-mediated lysis using flow cytometry to evaluate the regulatory role of holins over the lytic activities. Our data show that LytA is activated at the end of the lytic cycle and that its triggering results from holin-induced membrane permeabilization. In the absence of phage lysin, LytA is able to mediate bacterial lysis and phage release, although exclusive dependence on the autolysin results in reduced virion egress and altered kinetics that may impair phage fitness. Under normal conditions, activation of bacterial LytA, together with the phage lysin, leads to greater phage progeny release. Our findings demonstrate that *S. pneumoniae* phages use the ubiquitous host autolysin to accomplish an optimal phage exiting strategy.

2. INTRODUCTION

Streptococcus pneumoniae (pneumococcus), a common and important human pathogen, is characterized by the high incidence of lysogeny in isolates associated with infection [1,2]. Pneumococcal bacteriophages (phages) share with the majority of bacteriophages infecting other bacterial species the holin-lysin system to lyse the host cell and release their progeny at the end of the lytic cycle. Genes encoding both holins and lysins (historically termed endolysins) are indeed found in the genomes of all known pneumococcal phages [3-6]. Supporting this mechanism, a lytic phenotype in the heterologous *Escherichia coli* system was achieved only by the simultaneous expression of the Ejh holin and the Ejl endolysin of pneumococcal phage EJ-1 [3]. When these proteins were independently expressed, cellular lysis was not perceived. Similar results were shown for pneumococcal phage Cp-1, not only in *E. coli*, but also in the pneumococcus itself [4].

Phage lysins destroy the pneumococcal peptidoglycan network due to their muralytic activity, whereas holins have been shown in *S. pneumoniae* to form nonspecific lesions [3], most likely upon a process of oligomerization in the cytoplasmic membrane, as observed for the *E. coli* phage λ [7-9]. It was generally proposed that holin lesions allow access of phage lysins to the cell wall [10,11], as the majority of phage lysins, including the pneumococcal endolysins, lack a typical N-terminal secretory signal sequence and transmembrane domains [3]. However, recent evidence also highlights the possibility for a holin-independent targeting of phage lysins to the cell wall, where holin lesions seem to be crucial for the activation of the already externalized phage lysins [12-14]. Regardless of the mechanism operating in *S. pneumoniae* to activate phage lysins, holin activity compromises membrane integrity.

Pneumococcal cells present their own autolytic activity, mainly due to the presence of a powerful bacterial cell wall hydrolase, LytA (an *N*-acetylmuramoyl-L-alanine amidase), responsible for bacterial lysis under certain physiological conditions [15]. Although other bacterial species also encode peptidoglycan hydrolases, the extensive lysis after entering stationary phase caused by LytA is a unique feature of *S. pneumoniae*. Interestingly, LytA is translocated across the cytoplasmic membrane to the cell wall, where it remains inactive, in spite of the absence of a canonical N-terminal sequence signal [16]. In the cell wall, autolysin activities are tightly regulated by mechanisms that seem to be related to the energized state of the cell membrane. In fact, depolarizing agents are able to trigger autolysis in *Bacillus subtilis* [17,18], and bacteriocin-induced depletion of membrane potential triggers autolysis of some species of the genera *Lactococcus* and *Lactobacillus*, closely related to streptococci [19]. It is therefore possible that the holin-inflicted perturbations of the *S. pneumoniae* cytoplasmic

membrane upon the induction of the lytic cycle may trigger not only the lytic activity of the phage lysin, but also that of inactive LytA located in the cell wall. Accordingly, LytA could also participate in the release of phage particles at the end of the infectious cycle, especially considering its powerful autolytic activity. Previous studies have suggested a role for the host autolytic enzyme in the release of phage progeny [20,21], but in fact, the evidence is unclear and dubious, considering that the existence of phage-encoded lysins was unknown or very poorly understood and some of the experimental conditions used to show a role of LytA could have also affected the activity of the phage lysin [20].

To clarify the possible role of the bacterial autolysin in host lysis, we used the *S. pneumoniae* strain SVMC28, lysogenic for the SV1 prophage [1], which contains a typical holin-lysin cassette, and a different host strain lysogenized with the same SV1 phage. Our results show that LytA is activated by the holin-induced membrane disruption, just like the phage endolysin. In the absence of the endolysin, LytA is capable of mediating host lysis, releasing functional phage particles able to complete their life cycle. Still, sole dependence on LytA results in an altered pattern of phage release that may reduce phage fitness. Importantly, we also show that, together with the endolysin, the concurrent LytA activation is critical for optimal phage progeny release.

3. MATERIAL AND METHODS

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table II.1. *S. pneumoniae* strains SVMC28 and R36A were obtained from the Rockefeller University collection (A. Tomasz). R36AΔ*lytA* was kindly provided by S. Filipe. SVMC28 is a clinical isolate lysogenic for phage SV1. All *S. pneumoniae* strains were grown in a casein-based semisynthetic medium (C+Y) at 37°C without aeration [22] or in tryptic soy agar (Oxoid, Basingstoke, England) supplemented with 5% (v/v) sterile sheep blood and incubated at 37°C in 5% CO_2. Pneumococcal mutant strains were grown in the presence of 2 µg/ml erythromycin or 4 µg/ml chloramphenicol (Sigma, Steinheim, Germany), or both, as appropriate. *E. coli* strains were usually grown in LB medium (Difco, MD). When required, the medium was supplemented with 100 µg/ml ampicillin (Sigma, Steinheim, Germany), 1 mg/ml erythromycin, or 20 µg/ml chloramphenicol for plasmid selection. M9 minimal medium agar containing thiamine (1 mM; Sigma, Steinheim, Germany) was used for JM109 growth prior to the preparation of competent cells.

Antibiotic susceptibility

Chloramphenicol and erythromycin MICs were determined by Etest following the manufacturer's guidelines (AB Biodisk, Solna, Sweden). Susceptibility to novobiocin was tested with impregnated paper discs (Oxoid, Hampshire, England). PCR to detect the *cat* gene, which amplifies a 338-bp fragment internal to the gene, in the SVMC28 strain was performed using the primer pair CATd and CATr (Table II.2) [23].

DNA techniques

All routine DNA manipulations were performed according to standard methods [24]. The PCR primers are listed in Table II.2. Chromosomal DNA from *S. pneumoniae* and phage DNA were isolated similarly to previously described procedures [25-27]. Plasmids were prepared using either the High Pure plasmid isolation system or the Genopure plasmid Midi system (Roche, Mannheim, Germany), and PCR products and endonuclease digests were purified using the High Pure PCR product purification system (Roche, Mannheim, Germany). The enzymes used in the manipulation of DNA were purchased from MBI Fermentas (Vilnius, Lithuania). All

oligonucleotides were obtained from the Invitrogen Co. (Paisley, Scotland). Nucleotide sequences were analyzed using VECTOR NTI Deluxe (Invitrogen, Barcelona, Spain) software.

Construction of pneumococcal mutants by insertional deletion of *lytA* and *svl*

The mutant strain SVMC28Δ*lytA*, in which the *lytA* gene was deleted and replaced by the *erm*(B) gene, was constructed essentially as described previously [28]. First, *lytA*up (529 bp), the sequence encoding the upstream fragment of *lytA*, was amplified with the primers AFLYTA and ARLYTA from SVMC28 chromosomal DNA. The PCR product was digested with EcoRI and BamHI and inserted into the plasmid pGEM-3Z to generate pZ1. Next, *lytA*dw (509 bp), the sequence encoding the downstream fragment of *lytA*, was amplified from chromosomal DNA of SVMC28 using the primers BFLYTA and BRLYTA, and the PCR product was inserted as an XmiI-PaeI fragment into pZ1, generating pZ2. Lastly, the BamHI/ClaI fragment (2051 bp) from pJDC9, which contained the Erm^r cassette [*erm*(B) gene] [29], was cloned into the BamHI/XmiI-digested plasmid pZ2, yielding pZ3. Plasmid pZ3 contained the *erm*(B) marker flanked by the upstream and downstream regions of *lytA* and was used as a template for PCR with AFLYTA and BRLYTA to produce an aLTA fragment. After transformation of SVMC28 with aLTA, the deletion of *lytA* in the erythromycin-resistant SVMC28Δ*lytA* mutant was confirmed by PCR amplification and subsequent sequencing with primers DINF-D4 and ORF1-R3, external to AFLYTA and BRLYTA (supplementary data, Fig.II.S1A). An identical strategy was used to construct the mutant SVMC28Δ*svl*, in which *svl* was replaced by the *cat* gene. *svl*up (583 bp) and *svl*dw (477 bp) were PCR amplified from SV1 phage DNA using the primer pairs AFLYS1-ARLYS1 and BFLYS1-BRLYS1. *svl*dw contained 185 bp of *svl*. A CAT fragment (1,053 bp), containing the Cm^r marker (*cat*), was amplified from pEVP3 with BM18H and PEVP3-1R and cloned into pZ5 as a BamHI-XmiI fragment. aLS1 was produced with AFLYS1 and BRLYS1 from pZ6, and the resulting PCR product was used to transform the pneumococcal strain SVMC28. The deletion of *svl* in the chloramphenicol-resistant SVMC28Δ*svl* mutant was confirmed by PCR amplification and sequencing with primers 28HA37-R5 and 28HA37-X1, external to AFLYS1 and BRLYS1 (Fig.II.S1B). To construct the double-deletion mutant SVMC28Δ*svl*Δ*lytA*, the PCR fragment aLTA was used to transform SVMC28Δ*svl*. The chloramphenicol- and erythromycin-resistant strain SVMC28Δ*svl*Δ*lytA* was confirmed to carry deletions of the *lytA* and *svl* genes by the procedures described above (Fig.II.S1). For all PCRs, except those used as templates in sequencing reactions, the High Fidelity PCR Enzyme Mix kit (MBI Fermentas, Vilnius, Lithuania) was used. The amplicons (DNA products amplified by PCR) are described in Table II.1.

Transformation of *S. pneumoniae* and selection of transformants

Transformation of pneumococcal cells was carried out as described previously [28]. Transformants were selected on tryptic soy agar supplemented with 5% (v/v) sheep blood and chloramphenicol (4 μg/ml) and/or erythromycin (2 μg/ml).

Analysis of phage excision

The pulsed-field gel electrophoresis (PFGE) procedure for the visualization of extrachromosomal phage DNA was adapted from the method of Ramirez et al. [1], except that cells were harvested after a 2-h period of mitomycin C (MitC) (Sigma, Steinheim, Germany) treatment. The electrophoresis conditions were 6 V/cm, ramping of the pulse between 1 and 2 s, and a total running time of 16 h. The buffer was maintained at 14°C during the run.

Construction of lysogenized strains

The lysogenized strains were constructed using the laboratory strain R36A. SV1, obtained from wild-type SVMC28, was used to lysogenize strains R36A and R36AΔ*lytA*, resulting in strains R36AP and R36APΔ*lytA* (Table II.1). To obtain lysogenic strains defective in the phage lysin, R36A and R36AΔ*lytA* were infected with SV1Δ*svl* extract from SVMC28Δ*svl*. The resulting lysogens were named R36APΔ*svl* and R36APΔ*lytA*Δ*svl*, respectively (Table II.1). Phage infection was performed by phage plaque assays (see below). Agar was picked at the edges of plaques. The presence of both wild-type and mutant phages was tested for by PCR with primers for SV1 [AFLYS1 and ARLYS1 (Table II.2)]. MitC-induced lysis was performed on selected PCR-positive colonies. We considered that response to MitC was indicative of phage excision and consequently of successful prior lysogeny. The released phages were able to infect and lyse cells of the wild-type R36A strain, producing phage plaques and confirming that the strains were indeed lysogenic and that the phages were fully functional.

Lysis assays

Overnight cultures of wild-type, mutant, and lysogenized strains in C+Y supplemented with the appropriate antibiotics were diluted 1:100 in fresh medium (without antibiotics), and the cultures were grown until the optical density at 600 nm (OD_{600nm}) reached approximately 0.2 to 0.25. MitC was then added to a final concentration of 0.1 μg/ml to induce the lytic cycle [30]. Incubation was continued, and growth was monitored by the OD. Cultures treated with

deoxycholate (DOC) [0.04% (w/v)] and nisin (1 µg/ml) (Sigma, Steinheim, Germany) were grown to an OD_{600nm} of 0.4 and 0.2 to 0.25, respectively. All assays were carried out at least in duplicate. Lysis was expressed directly as the OD_{600nm} drop or as the percentage decrease in the OD_{600nm} relative to its maximal value (the OD value prior to lysis). The degree of lysis at each time point was calculated from the following equation: lysis extent (percentage) = 100 – (percentage of maximal OD).

Phage plaque assays

Plaque assays were performed as described elsewhere [31] with the following modifications: C+Y medium with 170 U catalase/ml agar was used, top agar was not added, and phage were applied in 10 µl aliquots directly on the soft agar (0.35%) with the indicator strain. Incubation was performed at 30°C. Lysogenic phages were induced with MitC. At specific times (between 40 and 180 min) after MitC treatment, cultures were filtered through a 0.45 µm-pore-size membrane, and the supernatant was stored at 4°C for a maximum of 24 h until it was used. To lysogenize the strains, cultures were filtered after total lysis (180 min). To eliminate the possibility that lysis of the indicator strain was caused by the bacterial products (e.g., bacteriocins) and not caused by phage infection, a fractionation of the culture medium was performed. Cultures treated for 180 min with MitC were prefiltered through a 0.45 µm-pore-size membrane, followed by filtering them with a 100 kDa mass-cutoff polyethersulfone membrane (Vivaspin 20 concentrator; Sartorius Stedim Biotech, Goettingen, Germany), which retained the SV1 phage but not proteins that could cause bacterial lysis, such as LytA, Svl, holins, and bacteriocins (mass < 100 kDa). Both the filtrate containing proteins of < 100 kDa and that containing the phage particles were used. Phage plaques were observed with a Leica MZ7.5 high-performance stereo microscope (Leica Microsystems, Germany).

Table II.1. Bacterial strains, plasmids, and DNA constructs used in this study.

Strain, plasmid or DNA construct	Relevant characteristics[a]	Use in this study	Source[b] or reference
S. pneumoniae			
SVMC28	Lysogenic for phage SV1; parental strain susceptible to Ery and Cm	Expression of LytA and Svl	[1]
SVMC28Δ*lytA*	SVMC28Δ*lytA*::*erm*(B); Ery^r	Expression of Svl, absence of LytA expression	SVMC28 X aLTA
SVMC28Δ*svl*	SVMC28Δ*svl*::*cat*; Cm^r	Expression of LytA, absence of Svl expression	SVMC28 X aLS1
SVMC28Δ*svl*Δ*lytA*	SVMC28Δ*svl*::*cat* Δ*lytA*::*erm*(B); Cm^r, Ery^r	Absence of LytA and Svl expression	SVMC28Δ*svl* X aLTA
CP1500	Nov^r	Donor of point markers, control in transformation assays	[32]
R36A	Laboratory strain; nonlysogenic; susceptible to Ery and Cm	Recipient for phage infection	Rockefeller University Collection
R36AΔ*lytA*	R36A*lytA*::(pJDC9)::*lytA*; Ery^r	Recipient for phage infection	S. Filipe
R36AP	R36A lysogenic for phage SV1	Expression of LytA and Svl	R36A infected with SV1
R36APΔ*lytA*	R36AΔ*lytA* lysogenic for phage SV1; Ery^r	Expression of Svl, absence of LytA expression	R36AΔ*lytA* infected with SV1
R36APΔ*svl*	R36A lysogenic for phage SV1Δ*svl*::*cat*; Cm^r	Expression of LytA, absence of Svl expression	R36A infected with SV1 with *svl* deleted
R36APΔ*lytA*Δ*svl*	R36AΔ*lytA* lysogenic for phage SV1Δ*svl*::*cat*; Cm^r, Ery^r	Absence of LytA and Svl expression	R36AΔ*lytA* infected with SV1 with *svl* deleted
E. coli			
DH5α	*lacZ*ΔM15	Recipient for pZ1-pZ3	Invitrogen
JM109	F´*lacI*^{q}ZΔM15	Recipient for pZ4-pZ6	
Plasmids			
pGEM-3Z	*lacZ*α; Amp^r	Cloning vector	
pZ1	pGEM-3Z::*lytA*up; Amp^r	Cloning vector	*lytA*up (AFLYTA, ARLYTA)
pZ2	pZ1::*lytA*dw; Amp^r	Cloning vector	*lytA*dw (BFLYTA, BRLYTA)
pZ3	pZ2::*erm*(B); Amp^r, Ery^r	Template for PCR product aLTA	
pZ4	pGEM-3Z::*svl*up; Amp^r	Cloning vector	*svl*up (AFLYS1, ARLYS1)
pZ5	pZ4::*svl*dw; Amp^r	Cloning vector	*svl*dw (BFLYS1, BRLYS1)
pZ6	pZ5::*cat*; Amp^r, Cm^r	Template for PCR product aLS1	CAT (BM18H, PEVP3-1R)
pEVP3	Cm^r	Source of *cat* gene; template for PCR product CAT	[33]
pJDC9	Ery^r	Source of *erm*(B) gene	[29]
Amplicons			
aLTA	*lytA*up::*erm*(B)::*lytA*dw	For insertion-deletion replacement for *lytA*	aLTA (AFLYTA, BRLYTA)
aLS1	*svl*up::*cat*::*svl*dw	For insertion-deletion replacement for *svl*	aLS1 (AFLYS1, BRLYS1)

[a] Ery^r, erythromycin resistance; Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance; Nov^r, novobiocin resistance.
[b] Strain construction by a cross carried out by using transformation is indicated as recipient X DNA donor. Primes used to amplify DNA fragments by PCR are indicated in parentheses.

Table II.2 - PCR primers used in this study.

Primer	Sequence (5´→3´)[a]	Recognition site
CATd	TTAGGYTATTGGGATAAGTTA	-
CATr	CATGRTAACCATCACAWACCAG	-
AFLYTA	AGCGAATTCGGCAGGATATAAGGGTGTTATC	EcoRI
ARLYTA	ATAGGATCCATTCTACTCCTTATCAATTAAAAC	BamHI
BFLYTA	GCAGTCGACTAATGGAATGTCTTTCAAATC	XmiI
BRLYTA	CAATAGCATGCGATATTCCTTTCACCTTTTTCC	PaeI
DINF-D4	GCAAAAGATCCTTCCTCTAGTTTC	-
ORF1-R3	CTTCACCATCAGCTCCCAAC	-
AFLYS1	AGCGAATTCAGGGGTTCTCTTACTGATGATC	EcoRI
ARLYS1	ATAGGATCCTCCCTATCGTCCTTTCCATGC	BamHI
BFLYS1	GCAGTCGACTGAAGACAGGCTGGGTCAAGTAC	XmiI
BRLYS1	CAATAGCATGCGCTATTTCCCAAGGTGCTGG	PaeI
BM18H	ATAGGATCCGGGTTCCGAGGCTCAACGTCAA	BamHI
PEVP3-1R	CGAGGTCGACGGTATCGATAAGCT	XmiI
28HA37-X1	TCAGGTTACTTGAAAAGGCAATAG	-
28HA37-R5	CAACGTCGCCGTTCTGTTGAATC	-

[a] Recognition sites are underlined.

Viability assays

Flow cytometry analysis of cultures treated with MitC was performed. As a control for cell death, the cultures were treated with nisin and DOC. All compounds used were filtered through a 0.22 µm-pore-size membrane before flow cytometry measurement. In these assays, selective overnight cultures of wild-type SVMC28 and the derived mutants strains were diluted 1:100 in fresh 0.22 µm-filtered C+Y and grown to the appropriate OD_{600nm}. Cells were collected immediately after exposure and at 20-min intervals during a 2-h period and then diluted in sterile-filtered 0.85% NaCl to a concentration of ~$1x10^6$ cells/ml. Cell viability was assessed by using the Live/Dead BacLight bacterial viability kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, 1.5 µl of Syto 9 (green fluorescent nucleic acid stain; 3.34 mM) at a final concentration of 5 µM and 1.5 µl of propidium iodide (PI) (red fluorescent nucleic acid stain; 2 mM) at a final concentration of 3 µM were added to each 1-ml diluted sample of cells. PI stock solution was diluted to 2 mM in sterile-filtered distilled H_2O immediately prior to staining. The samples were then incubated at room temperature in the dark for 20 min and analyzed on a Partec CyFlow space flow cytometer (Partec GmbH, Münster, Germany) with 488-nm excitation from a blue solid-state laser at 50 mW. Forward scatter (FSC), side scatter (SSC), and two fluorescence signals were measured. Green fluorescence, indicating the population of live cells (nonpermeabilized cytoplasmic membranes) was detected in the FL1 channel, and red fluorescence, indicating the population of dead cells (permeabilized cytoplasmic membranes), was detected in the FL3 channel. Optical filters were set up so that FL1 measured at 520 nm and FL3 measured above 610 nm. The sample analysis rate was kept below 1000 events/s. The trigger was set for the FSC

channel, and the combination of FSC and SSC was used to discriminate bacteria from the background. Twelve thousand events were collected for each sample taken. Data were collected and analyzed using FloMax software (Partec GmbH, Münster, Germany). Assays were carried out at least in duplicate.

Cell viability was also assessed by fluorescence microscopy to confirm staining by the different strains after MitC treatment. Syto 9/PI-labeled cell suspensions were microscopically analyzed 40, 80, and 120 min after MitC addition with a Zeiss Axiovert 200 M microscope (Carl Zeiss, Germany) equipped with a 100-W halogen lamp, the appropriate excitation and emission filters for Syto 9 and PI [excitation wavelengths, 450 to 490 nm (Syto 9) and 540 to 552 nm (PI); emission wavelengths, 515 to 565 nm (Syto 9) and > 590 nm (PI)], a Plan Apochromat 63X/1.4 objective lens, and a CoolSnap HQ charge-coupled device camera (Roper Scientific Photometrics, Tucson, AZ). Fluorescence photographs were acquired with Metamorph software (version 6.1r0).

Statistical analysis

Differences in the mean values of the lysis extent between strains were analyzed by a Student *t* test. For all comparisons, a *P* value of < 0.05 was considered to represent statistical significance; 95% confidence intervals for the average of the OD measurements from different experiments were calculated based on the Student *t* distribution.

Nucleotide sequence accession number

The DNA sequence of the 1.719 kb SV1 lytic cassette has been assigned GenBank accession number FJ765451.

4. RESULTS

The prophage SV1 lytic cassette and construction of the lysin mutants

Strain SVMC28, an *S. pneumoniae* clinical isolate lysogenic for the inducible phage SV1, was selected for study [1]. The SV1 lytic cassette is localized downstream of the structural cluster and adjacent to the *attP* site in the SV1 genome, similarly to other pneumococcal lytic cassettes, and shows a high nucleotide sequence identity to the pneumococcal phage MM1 [5]. In addition, it exhibits a typical three-component organization in which the two open reading frames encoding putative holins (Svh1 and Svh2) precede the endolysin gene, which encodes a putative amidase (Svl). Attempts to clone *svh1* and *svh2* open reading frames in *E. coli* resulted in loss of viability, strongly indicating that these proteins correspond to holins (data not shown).

In order to construct *S. pneumoniae* mutants without bacterial and phage lytic activities, the *lytA* and *svl* genes were eliminated by insertion-deletion in the clinical isolate SVMC28 (Table II.1 and supplementary data, Fig.II.S1). Elimination of the lytic genes did not alter the growth rates of the mutants. In fact, all strains displayed growth curves indistinguishable from those of the parental strain, indicating that the modifications introduced had no significant impact on pneumococcal physiology. As expected, the *lytA*-deficient strains SVMC28Δ*lytA* and SVMC28Δ*svl*Δ*lytA* were greatly resistant to autolysis in stationary phase (data not shown) and when treated with nisin or DOC (see Fig.II.3). In these cases, only 30% to 45% lysis occurred 280 min after nisin or DOC addition, which was far less than that exhibited by the wild-type strain SVMC28 (close to 95%). The phage lysin played no role in the response to these stimuli, since SVMC28Δ*lytA* and SVMC28Δ*svl*Δ*lytA* showed similar lytic phenotypes. Thus, residual lysis was probably caused by other pneumococcal autolysins, such as LytC, which is also responsible for lysis in stationary phase but with a lower activity than LytA at 37°C [34,35]. The elimination of *svl* in the SVMC28Δ*svl* mutant did not alter the lytic response upon nisin and DOC treatment, which was characterized by the same lysis rate as the wild-type strain (see Fig.II.3), confirming that LytA is fully functional in this mutant strain.

Phenotypic evaluation of phage-induced lysis

To investigate the possible role of the autolysin LytA in phage release in *S. pneumoniae*, we started by comparing the lytic phenotypes of the parental and mutant strains after induction of the SV1 lytic cycle with MitC. As indicated in Fig.II.1A, SVMC28Δ*lytA* cultures showed pronounced lysis 80 min after MitC addition, in contrast to the control untreated

cultures. This is consistent with the known role of phage lysins in bacterial cell wall degradation to allow the release of new phage particles. Remarkably, in the mutant lacking endolysin (SVMC28Δ*svl*), lysis was unequivocally detected after treatment with MitC, clearly demonstrating that LytA is activated after prophage induction.

Analysis of the MitC-treated double mutant SVMC28Δ*svl*Δ*lytA*, which lacks LytA and Svl, revealed negligible lysis with a maximum of 35% lysis at 180 min (Fig.II.1A), which may reflect the activity of LytC [35]. A PFGE analysis of the total DNA showed no considerable changes in prophage excision compared to the wild-type in SVMC28Δ*svl* and SVMC28Δ*svl*Δ*lytA* mutant strains, with all MitC-treated cultures showing free phage DNA (~33 kb), which was not detected in the untreated control cultures (Fig.II.1B). This was also observed for the *lytA*-deficient mutant SVMC28Δ*lytA* (data not shown). The extra fragments visualized below the chromosome are concatemers of the phage genome, as already demonstrated for SV1 [1]. These observations clearly demonstrate that, similarly to the wild-type, in all mutant strains, the phage successfully initiated the lytic cycle with DNA excision and replication, indicating that the observed lysis after MitC addition was phage mediated.

To test whether this newly discovered role of LytA was independent of the bacterial genetic context, we characterized the lysis of similar mutants generated in the R36A background by lysogenizing the laboratory strains R36A and R36AΔ*lytA* with phages SV1 and SV1 with *svl* deleted (SV1Δ*svl*) (Table II.1). As depicted in Fig.II.1C, MitC induction of phage excision in the endolysin-deficient R36APΔ*svl* strain resulted in pronounced lysis, confirming the activation of LytA. Thus, independently of the host genetic background, LytA mediates phage-induced lysis of the host cell in the absence of the endolysin.

It is noteworthy that the lysis rates of the Δ*svl* mutant were similar to those of the Δ*lytA* mutant in both SVMC28 and R36A backgrounds (Fig.II.1A and C), indicating that the exclusive presence of the autolysin LytA in the absence of any phage lytic enzyme is sufficient for an accentuated decrease in OD at the end of the lytic cycle. However, the bacterial autolysin appears not to influence the lysis rate or the lysis extent promoted by Svl, since these parameters were similar between the SVMC28 and R36AP strains (containing both enzymes) and the respective Δ*lytA* mutants, in which just the Svl activity is present (Fig.II.1A and C). Nevertheless, for R36A in the absence of LytA (strain R36APΔ*lytA*), the lysis timing was delayed. Thus, in contrast to the experiment performed in the SVMC28 genetic background, in the R36A strain, LytA is essential to control the exact timing of lysis.

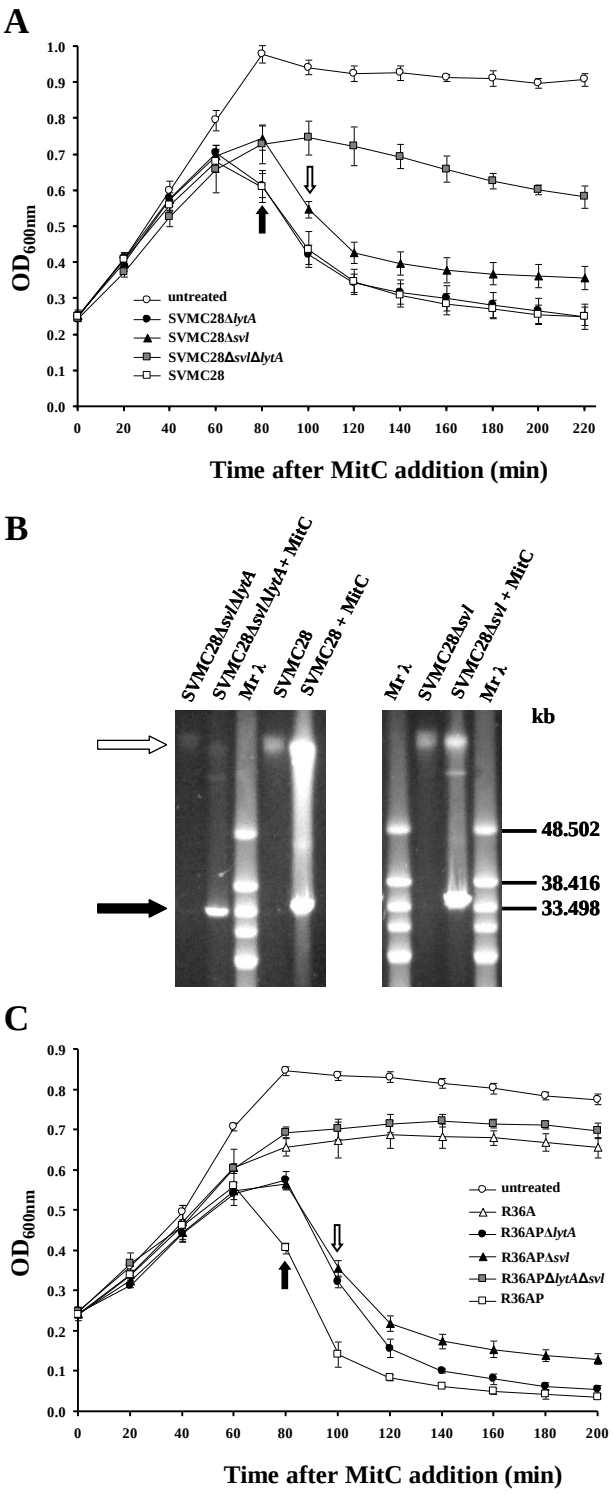

Figure II.1. Participation of bacterial and phage lysins in pneumococcal phage-induced lysis. (**A**) Lysis profiles of SVMC28 strains. Wild-type SVMC28 and the derived mutants were grown to an OD_{600nm} of 0.2 to 0.25, and 0.1 µg/ml of MitC was added to induce phage excision (0 min). (**B**) PFGE analysis of extrachromosomal phage DNA induced with MitC. Total DNA was isolated from cultures of SVMC28, SVMC28Δ*svl*, and SVMC28Δ*svl*Δ*lytA* treated with MitC or left untreated (control). The preparations were separated by PFGE. The white arrow indicates the bacterial chromosome (about 2.2 Mb), while the black arrow indicates phage DNA. Similar PFGE profiles were obtained for SVMC28Δ*lytA* (data not shown). $M_r\lambda$, lambda ladder PFGE marker (New England Biolabs, Beverly, MA). (**C**) Lysis profiles of lysogenized R36A strains. R36AP, R36APΔ*lytA*, R36APΔ*svl*, and R36APΔ*lytA*Δ*svl* were grown to an OD_{600nm} of 0.2 to 0.25, and 0.1 µg/ml of MitC was added to induce phage excision (0 min). In panels A and C, the arrows represent the times at which lysis started: 80 min after MitC addition for SVMC28, SVMC28Δ*lytA*, and R36AP (black arrows) and 100 min for SVMC28Δ*svl*, R36APΔ*svl*, and R36APΔ*lytA* (white arrows). The untreated SVMC28 and R36AP cultures are representative of the growth curves of all untreated strains. The results are averages of a minimum of four independent experiments, and 95% confidence intervals are indicated.

In both genetic backgrounds, exclusive reliance on LytA for lysis resulted in a 20-min delay relative to when both phage and host lysins were present (Fig.II.1A and C). This delay was not a reflection of a difference in growth rates, as the doubling time of the Δ*svl* mutants was comparable to those of the SVMC28 and R36AP strains. In addition, a substantial reduction of the total lysis percentage in the Δ*svl* mutants relative to the strains carrying both bacterial and phage lysins was also observed [at 180 min, SVMC28Δ*svl* and SVMC28, $P < 10^{-4}$ (Fig.II.1A) and R36APΔ*svl* and R36AP, $P < 10^{-4}$ (Fig.II.1C)].

Collectively, these results suggest that in the absence of phage endolysin, although LytA is able to mediate bacterial lysis, already assembled phage particles may be retained inside the bacterial host for a longer time, and that relying exclusively on the host autolysin could have an important impact on the quantity of phage particles released.

Phenotypic assessment of phage release

Although the different lytic phenotypes provide clues about the changes in the phage particles released, the measurement of phage production was essential to determine if a significant difference in the release of phage progeny was observed. A phage plaque assay, using strain R36A as an indicator, was therefore performed using the supernatant of the MitC-induced strains SVMC28 and R36AP and the corresponding mutants at different time points. When the R36A lysogens (with phage already adapted to infect R36A) were used, the plating efficiency was improved relative to the phage obtained directly from SVMC28, providing excellent conditions to explore the differences attributed to LytA in virion release.

In the absence of endolysin, phage plaques were clearly observed, indicating that LytA by itself allows the release of functional phages capable of completing their life cycle (Fig.II.2A). However, in accord with the delayed lysis timing, phage plaques obtained for R36APΔ*svl* were observed only from 100 min onward, whereas for R36AP, phage plaques were already visible 80 min after MitC addition, when culture lysis was detected (Fig.II.2A). In agreement with the reduced lysis mediated exclusively by LytA relative to that observed in the presence of both host and phage lysins, the number of phage released when only LytA was present was also significantly diminished (at 180 min, $P < 10^{-5}$) (Fig.II.2D). Thus, these results support previous observations and confirm that lysis strictly dependent on LytA may severely impair phage fitness by reducing phage progeny release and delaying its timing.

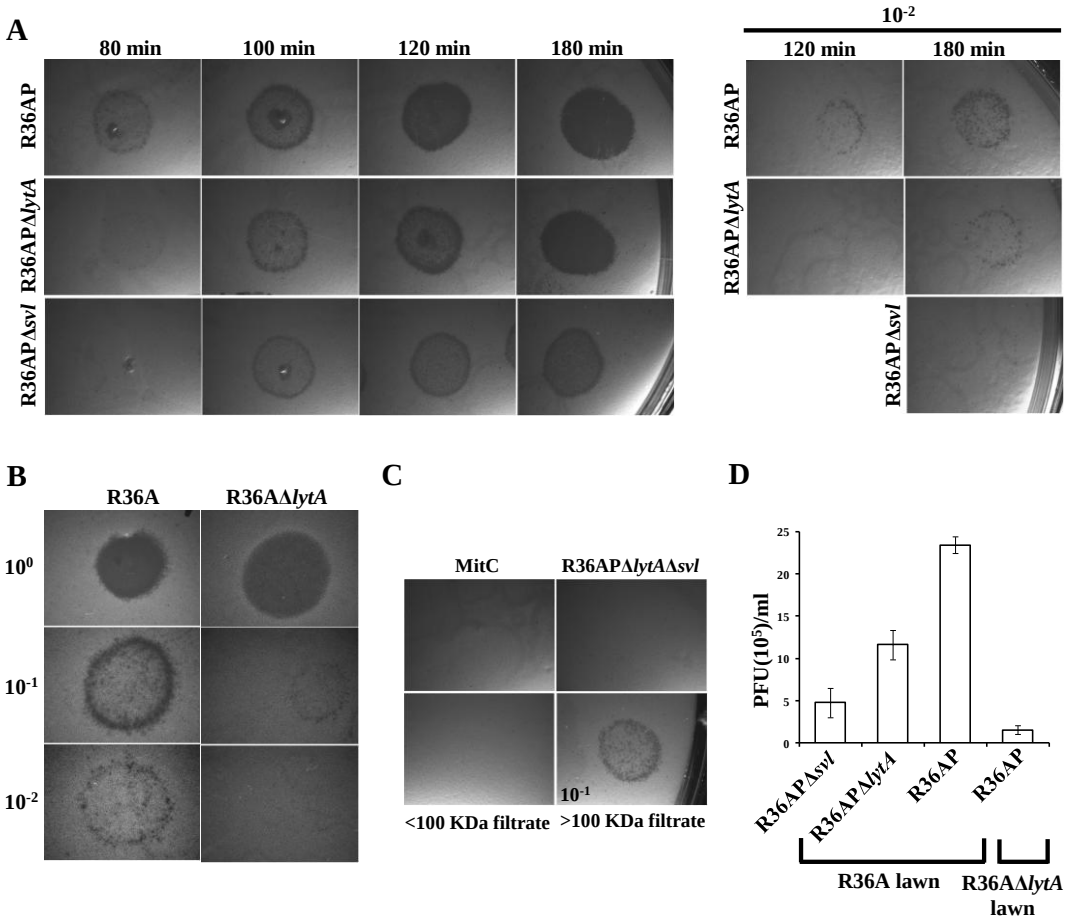

Figure II.2. Participation of bacterial and phage lysins in phage release. (**A**) Phage release patterns. The culture media of R36AP, R36APΔ*svl*, R36APΔ*lytA*, and R36APΔ*lytA*Δ*svl* treated with MitC were filtered (0.45 µm) at 20-min intervals after the start of lysis in the R36AP strain, and the supernatants were used directly in phage plaque assays of the indicator strain R36A. The time after MitC addition is indicated. The results are representative of three independent experiments. (**B**) Phage plaque assay using indicator strains differing only in the presence or absence of LytA. The supernatant of the culture medium of R36AP was collected 180 min after MitC treatment and used on indicator strains R36A and R36AΔ*lytA*. The results are representative of three independent experiments. (**C**) Indicator lawn lysis is due to phage induction. As a control, R36A indicator lawns were exposed to C+Y medium with MitC at the same concentration used for phage induction. For R36APΔ*lytA*Δ*svl*, the supernatant collected 180 min post-MitC addition produced no phage plaques. The supernatant of an R36AP culture treated for 180 min with MitC was filtered through a 100 kDa-cutoff membrane to retain phages while eliminating small proteins. The < 100 kDa filtrate showed no phage plaques, unlike the retained fraction (> 100 kDa filtrate; 10^{-1} dilution), demonstrating that the plaques were due to phage and were not due to the action of bacterial and phage lysins, holins, or bacteriocins (mass < 100 kDa), which can cause cell lysis. (**D**) Comparison of the numbers of PFU per ml detected upon phage induction. The numbers of PFU per ml were determined for strains R36AP, R36APΔ*svl*, and R36APΔ*lytA* after 180 min of MitC treatment on indicator lawns of strains R36A and R36AΔ*lytA*, as indicated. Averages and 95% confidence intervals are indicated. For R36A in the indicator lawn, the comparisons were between supernatants obtained from R36APΔ*lytA* and R36AP ($P < 10^{-4}$), R36APΔ*svl* and R36APΔ*lytA* ($P < 10^{-3}$), and R36APΔ*svl* and R36AP ($P < 10^{-5}$). When the R36AP supernatant was plated on indicator lawns of strains R36A and R36AΔ*lytA*, *P* was < 10^{-9}. Magnification (A to C), x6.3 or x8.0.

Surprisingly, the bacterial-lawn clearance due to infection with supernatant from R36APΔ*lytA* was different from that due to R36AP (Fig.II.2A). The lysis delay between

R36APΔ*lytA* and R36AP prevented a direct comparison of the phage plaques obtained at most of the time points studied. Nevertheless, at 180 min, when the two lytic processes reached indistinguishable and approximately stable lysis extents (Fig.II.1C), the bacterial lawn clearance due to infection with R36APΔ*lytA* supernatant was much less pronounced than that due to infection with R36AP supernatant (Fig.II.2A), resulting from fewer phage being released ($P < 10^{-4}$) (Fig.II.2D). Similarly, for the SVMC28 genetic background, in the absence of LytA, the bacterial clearance due to phage infection was always less marked at any given time than that observed for the wild-type (data not shown). This was also unexpected, since the lysis of SVMC28Δ*lytA* and the wild-type was characterized by the same overall extent, timing, and rate (Fig.II.1A). Thus, one would expect that the numbers of phage released would be equivalent at any given point of the lytic process for those strains.

Thus, these data reveal a negative impact on phage release in the absence of LytA, indicating that the bacterial autolysin, together with the phage endolysin, maximizes progeny release, contributing substantially to this process. In fact, another set of experiments further established this role. When the same phage preparation obtained after total lysis of MitC-treated R36AP was used to infect strains R36A and R36AΔ*lytA* as indicators (differing only in the presence of a functional LytA), fewer phage plaques were obtained for R36AΔ*lytA* ($P < 10^{-9}$) (Fig.II.2B and D), demonstrating the importance of the presence of the host lysin for plaque formation and supporting its role in phage release.

Membrane permeabilization and cell lysis

It has been shown for some bacteria that holins strictly control the lysis timing by disrupting the bacterial cytoplasmic membrane, which results in the triggering of the endolysin activity [7,10,12,14,36]. In *S. pneumoniae*, phage endolysins depend on holin activity for efficient peptidoglycan degradation [3,4], but whether the timing of lysis, and thus the endolysin activity, is controlled by holins has yet to be determined. Given the observation that LytA is able to mediate phage-induced lysis, we tested if the permeabilization and consequent depolarization of the cytoplasmic membrane caused by the holins [7,9] is responsible for the activation of both lysins.

To address this issue, a real-time flow cytometry analysis of bacterial viability based on membrane integrity was performed after phage induction. In this assay, using a Live/Dead Bac-Light bacterial viability kit (Invitrogen, Carlsbad, CA), cells with damaged membranes (the dead population) allow the uptake of PI, fluorescing red (FL3 channel), whereas undamaged cells (the live population) internalize only the Syto 9 dye, fluorescing green (FL1 channel). The first

step consisted of setting gates that differentiated between the two populations. As a control for cell death, we used the antimicrobial agent nisin, which, by inserting into the cytoplasmic membrane, causes irreparable membrane damage and triggers pneumococcal LytA activity (Fig.II.3A) [37]. After 10 min, before complete lysis occurred in the wild-type culture, the untreated and the nisin-treated cells formed two well-defined and distinct populations (Fig.II.3B). Therefore, the gates representing damaged (R2) and undamaged (R3) cells were constructed using a 1:1 mixture of untreated and nisin-treated cells. Gating with R2 and R3 revealed that 99% of the untreated cells remained intact compared with less than 0.2% of the nisin-treated cells (Fig.II.3B). To assess the robustness of the constructed gates, we used the detergent DOC, a well-known trigger of LytA activity [15,38], which is assumed to have membrane-permeabilizing properties. In this experiment, the LytA-lacking mutant SVMC28Δ*svl*Δ*lytA* was used to avoid immediate lysis (Fig.II.3C and D). The untreated cells fit entirely into gate R3, and immediately after DOC addition, the cells were distributed almost exclusively in gate R2 (Fig.II.3D). Similar results were obtained with strain SVMC28Δ*lytA* (data not shown). Therefore, the chosen gates allowed complete differentiation of damaged cell populations due to other membrane perturbations, in addition to those induced by nisin.

The validated gates were then applied to determine the proportions of viable and permeabilized populations in wild-type and mutant cultures treated with MitC. Depending on the strain analyzed, different fluorescence patterns emerged. For the double mutant SVMC28Δ*svl*Δ*lytA*, a shift of the bacterial population toward increased red fluorescence intensity (R3 to R2) was observed, indicating that the cells were becoming permeabilized with increasing time after phage induction (Fig.II.4). In fact, after 120 min of phage induction, almost all cells were within the R2 gate. This feature was not observed for the wild-type strain (Fig.II.4) or the SVMC28Δ*lytA* strain (Fig.II.S2). At 80 min, membrane integrity was already compromised in a significant fraction of the SVMC28Δ*svl*Δ*lytA* bacterial population, precisely when lysis was evident in the wild-type (Fig.II.1A). In contrast, in both the SVMC28Δ*lytA* and wild-type strains, the majority of the population detected was viable, with the holin-damaged fraction corresponding to about 7% (Fig.II.4 and S2). This can be attributed to rapid lysis upon holin membrane permeabilization. The compartment of damaged cells that had not yet lysed was therefore very sparsely populated due to the extremely fast lysis triggered by holin activity. These data provide definite evidence that holin-induced membrane lesions trigger the lytic activity of the phage endolysin.

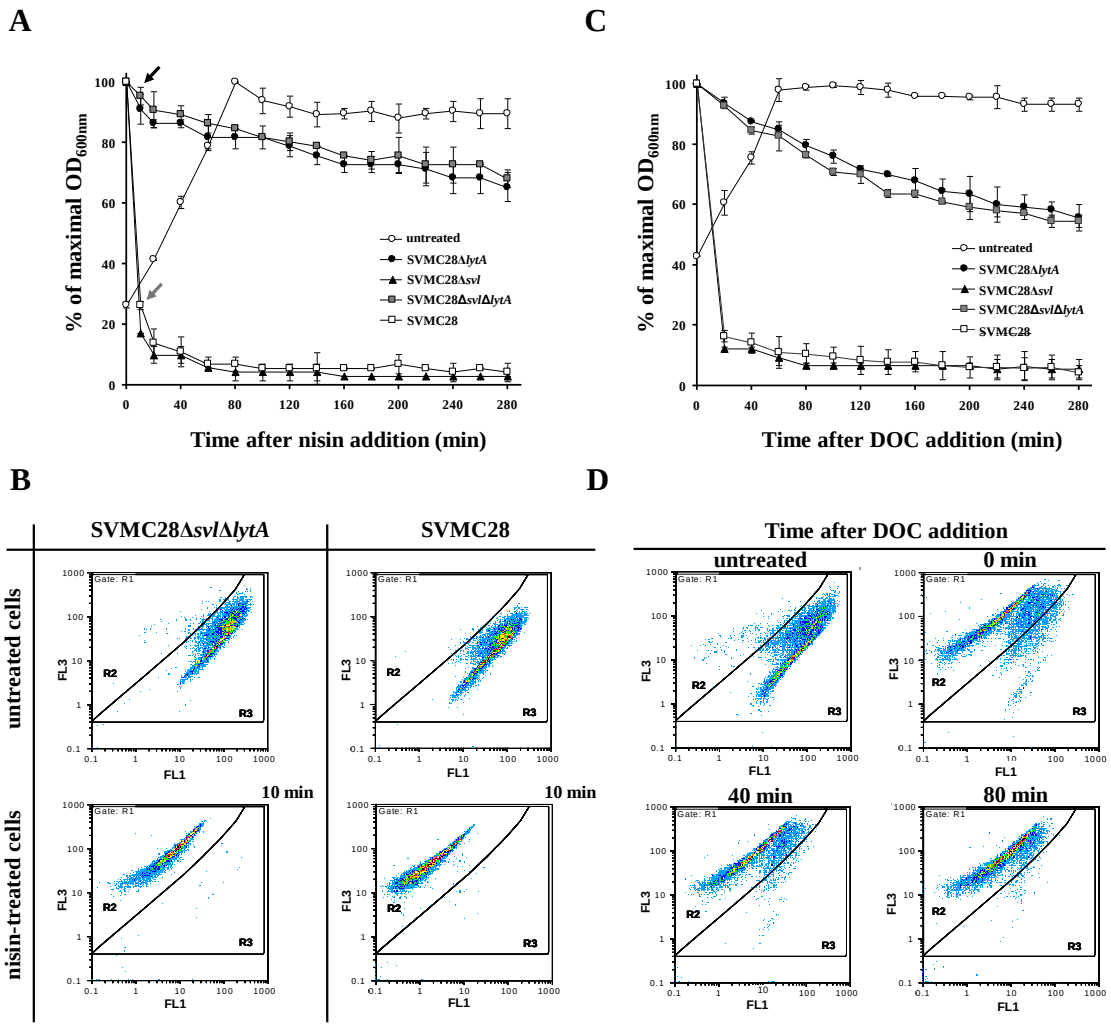

Figure II.3. **Kinetics of nisin- and DOC-triggered lysis of wild-type *S. pneumoniae* SVMC28 and mutants and corresponding flow cytometry analysis**. (**A**) Kinetics of nisin-triggered lysis. Nisin at a final concentration of 1 µg/ml was added to cultures at an OD_{600nm} of 0.2 to 0.25, and the OD was monitored. The arrows indicate the times at which nisin-treated cultures were harvested for flow cytometry analysis (10 min). (**B**) Flow cytometry analysis of wild-type SVMC28 and SVMC28Δ*svl*Δ*lytA* after nisin treatment. Exponentially growing cells were treated with nisin or left untreated (panel A), stained with a mixture of Syto 9 and PI, and analyzed on a flow cytometer. Similar analysis patterns were obtained for SVMC28Δ*lytA* and SVMC28Δ*svl* (data not shown). Gates R2 and R3 differentiated between damaged and undamaged cell populations, respectively, and were designed over gate R1, which included the total stained population. The results are representative of a minimum of two independent experiments. (**C**) Kinetics of DOC-triggered lysis. DOC at a final concentration of 0.04% (w/v) was added at time zero to cultures in mid-exponential phase (OD_{600nm}=0.4), and the turbidity was monitored. (**D**) Flow cytometry analysis of SVMC28Δ*svl*Δ*lytA* exposed to DOC. Exponentially growing cells were treated with DOC at 0.04% (w/v). Culture samples were collected at 0, 40, and 80 min after DOC addition (panel C), stained with a mixture of Syto 9 and PI, and analyzed on a flow cytometer. As a control, the same cells were left untreated. Gate definitions were as for panel A. The results are representative of a minimum of two independent experiments. In panels A and C, the results presented for each strain correspond to the mean value of at least two independent assays, and 95% confidence intervals are indicated.

We then went on to analyze by flow cytometry the mutant SVMC28Δ*svl* after MitC treatment to test the hypothesis that LytA may also be activated by the same holin lesions that induce Svl endolysin activation. In line with the observed lysis caused by LytA, the SVMC28Δ*svl*

pattern resembled that of the wild-type strain, with a large population of intact cells (gate R3) and a smaller fraction of damaged cells (gate R2) at every time point after MitC phage induction (Fig.II.4). Contrary to the large reservoir of dead bacteria observed in SVMC28Δ*svl*Δ*lytA*, the fraction of damaged cells was substantially smaller in SVMC28Δ*svl*, as the population driven into the R2 gate by the holins underwent lysis, becoming undetectable by flow cytometry. Thus, membrane permeabilization caused by the holins is responsible for triggering LytA activity. However, in SVMC28Δ*svl*, a larger population of membrane-damaged cells (gate R2) was clearly visible from 80 min onward, in contrast to the residual fraction observed in the wild-type (Fig.II.4). This observation is in agreement with the previously observed lysis delay mediated by LytA in the absence of endolysin. In this case, as the time between holin action and LytA-induced lysis was more prolonged, a greater percentage of holin-permeabilized but still unlysed cells was detected than with the wild-type. Besides the well-defined population of membrane-permeabilized cells, exclusively distributed in the R2 gate, there was also a population located between the R2 and R3 gates. This mixed population was also observed in SVMC28Δ*svl*Δ*lytA*, and since it was increasingly found in R2, it may correspond to chains containing both damaged and undamaged cells, where the number of damaged cells increased with time. This was indeed confirmed by fluorescence microscopy. As shown in Fig.II.5, the number of PI-stained cells within the chains increased with time after SV1 induction. For instance, at 80 min, when the total population was almost evenly distributed between R2 and R3 (Fig.II.4), approximately half of most chains in fact consisted of damaged, PI-stained cells (Fig.II.5C). In addition, these observations also confirm the increased permeabilization perceived from flow cytometry analysis. In line with these data, in the SVMC28Δ*svl* flow cytometry profile, more chains were observed with a mixture of damaged and undamaged cells than in the wild-type (Fig.II.4). This persistence of chains, which are not promptly dispersed by cell lysis, is caused by the delayed LytA-induced lysis of holin-damaged cells.

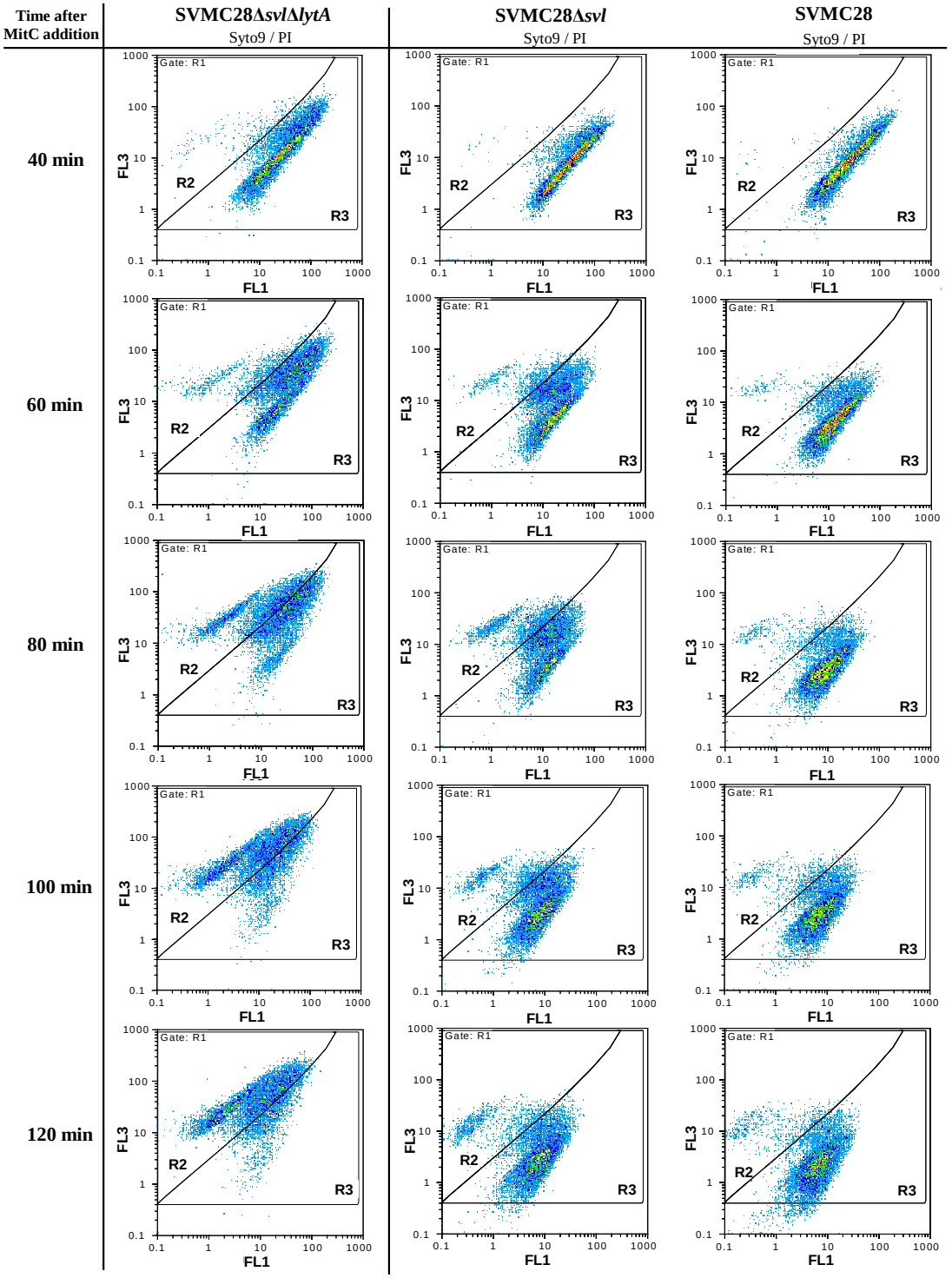

Figure II.4. Effect of phage holin activity on *S. pneumoniae* cell membrane permeabilization. Cultures of SVMC28, SVMC28Δ*svl*Δ*lytA*, and SVMC28Δ*svl* were treated with MitC and tested for membrane permeabilization at various times by flow cytometry analysis using a mixture of Syto 9 and PI staining (Live/Dead BacLight bacterial viability kit; Invitrogen, Carlsbad, CA). Experimentally defined gates R2 and R3 were used to differentiate between damaged and undamaged cell populations and were designed over gate R1, which included the total stained population (Fig.II.3). The left column shows a shift in the Syto 9/PI staining pattern through time after phage induction of strain SVMC28Δ*svl*Δ*lytA*, which lacks both the phage endolysin and the *S. pneumoniae* autolysin, LytA. In the presence of lytic enzymes (middle and right columns), a different scenario was observed, with only a few damaged cells detected. The data are from a representative experiment of a minimum of three independent experiments.

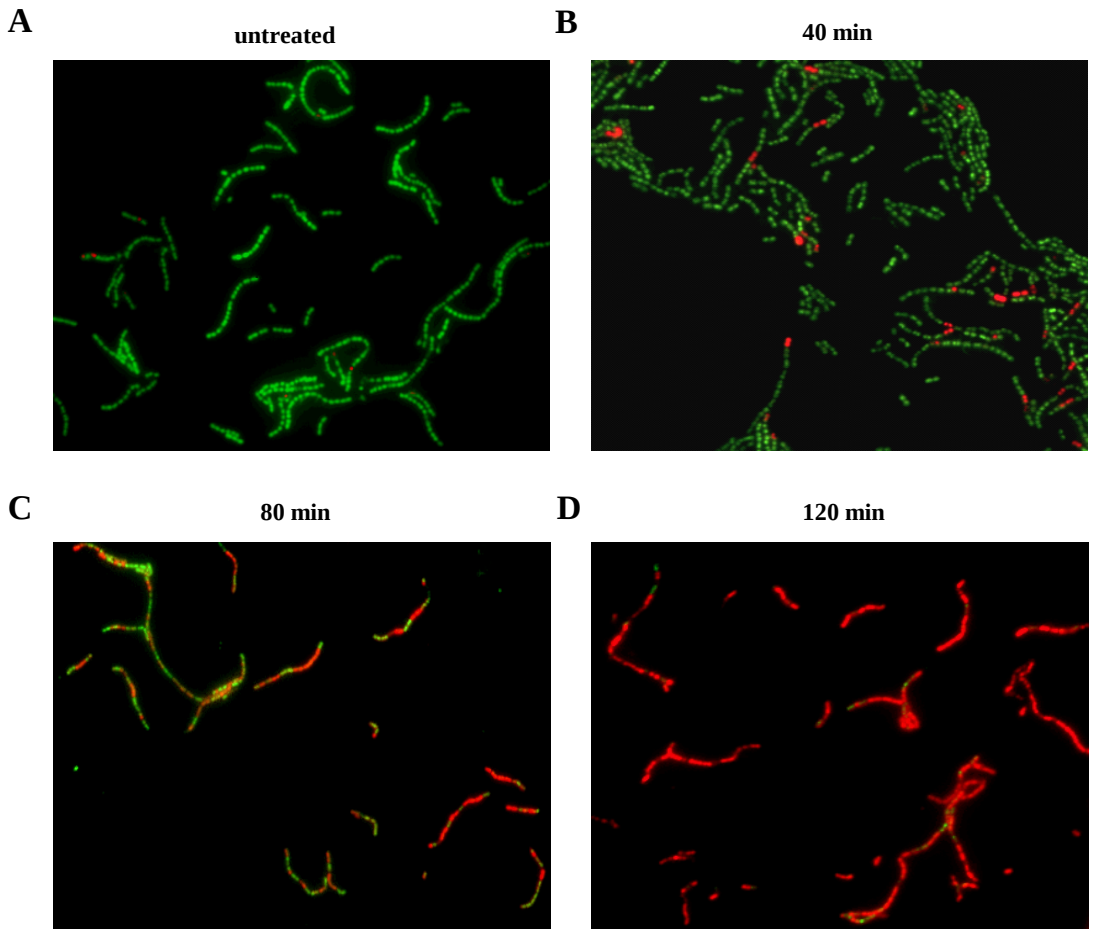

Figure II.5. Fluorescence microscopy analysis of Syto 9/PI-stained strain SVMC28Δ*svl*Δ*lytA* after MitC phage induction. SVMC28Δ*svl*Δ*lytA* culture samples were collected at 40, 80, and 120 min after the addition of 0.1 µg/ml MitC, stained with a mixture of Syto 9 and PI, and visualized on a fluorescence microscope (magnification, x630). As a control, the same cells were not treated with MitC. Different fluorescence patterns were clearly detected. (**A**) The untreated control corresponds mostly to bacteria exclusively stained with Syto 9. (**B**) After 40 min of phage induction, PI stained a few cells (dead cells), although the majority of cells stained only with Syto 9, indicative of intact membranes. (**C**) Eighty minutes after phage induction, almost half of the cells were stained with PI, with chains containing a mixture of PI- and Syto 9-stained cells. (**D**) After 120 min of phage induction, PI stained almost every cell. The chains showed few bacteria stained only with Syto 9. Each panel is from a representative experiment of four independent assays.

5. DISCUSSION

The holin-lysin strategy to release phage progeny through host lysis is the most widespread system in nature [39] and appears to be present in every *S. pneumoniae* phage [3-5]. In this system, phage endolysins ultimately destroy the host envelope, allowing the escape of fully assembled virions, and therefore, this phage-encoded function is essential [3,4,11]. However, the presence in *S. pneumoniae* of the powerful autolytic amidase LytA, which both structurally and functionally closely resembles pneumococcal phage endolysins [1,40-43], raises the possibility that it could play an important role in phage-mediated lysis. Since holins, the other protein components of phage holin-lysin systems, form lesions in the host membrane [3,7,9] and membrane depolarization leads to autolysis in *B. subtilis* [17], it is tempting to hypothesize that cell wall-resident LytA could be activated by the holin-induced lesions. Although it has been suggested that the pneumococcal autolytic enzyme LytA contributes to phage release, convincing evidence has never been provided [20,21]. In the earlier reports, some experimental conditions used to analyze the role of LytA (e.g., culture transfer after Dp-1 infection to medium containing ethanolamine instead of choline) inhibit autolysin activity, as indicated by the authors [20], but also inhibit the activity of phage lysins, which depend on choline for proper function in the majority of phages, including Dp-1. Thus, the inhibition of phage release could be attributed to inhibition of the Dp-1 endolysin activity. On the other hand, LytA was essential for lysis of strain R6 at a low multiplicity of infection (<1) of the virulent phage Dp-1, since no lytic phenotype was observed in the derived *lytA*-deficient strain, regardless of an evident role of the endolysin at a high multiplicity of infection (>1) in the strain lacking LytA [20,21]. Given those inconsistencies, we set out to clarify the role of the bacterial autolysin in host lysis and release of newly assembled phage particles, using SV1, a lysogenic pneumococcal phage that carries a typical lytic cassette encoding putative holin (Svh) and lysin (Svl) activities.

The data presented here reveal unambiguously that LytA is activated during pneumococcal phage-mediated lysis. In the absence of endolysin, this extremely powerful autolysin is able to mediate extensive host lysis that actually results in the release of a large number of fully functional phage capable of infecting other hosts. Thus, pneumococcal phages are able to use the bacterial autolysin LytA to exit from the host cell, completing their life cycle, in contrast to all other phages relying on a holin-lysin system. In the overwhelming majority of phages studied so far, mutants in the genes encoding endolysins are absolutely incapable of host lysis, trapping the phage progeny within the host cell [39]. In T7 and T4 *E. coli* phages, artificial deletion of the endolysin did not prevent host lysis [44]. However, in these

unusual cases, the phages evolved to use another protein with muralytic activity encoded in their genomes, whose native function is to assist in the initial stages of infection to allow entry of the phage genome into the host cytoplasm [44]. Dependence on lytic factors of cellular origin to disrupt the infected cell was indeed demonstrated only for phage PM2 of *Pseudoalteromonas* [45]. Still, this phage does not encode an endolysin in its genome to autonomously achieve bacterial-host lysis and uses a novel system different from the typical holin-lysin strategy for progeny release [45].

If LytA is activated upon phage induction, leading to productive lysis in the absence of phage endolysins, what part does LytA play in the overall process under physiological conditions? From the lytic phenotypes, it seems that LytA activation does not contribute significantly to endolysin-mediated lysis (except perhaps for earlier lysis in the R36A bacterial background). Given these observations, we hypothesized that LytA activation is not crucial to accomplish efficient phage progeny release and is merely a side effect of the induction of the phage lytic system. However, the results from the phage plaque assays point to a different and more complex scenario. Phage release achieved by Svl endolysin is maximized by LytA, since the number of phage particles released is diminished in the absence of bacterial autolysin. This was observed both in lysogenic SVMC28 and lysogenized R36A strains. Interestingly, in the R36A genetic background, the absence of LytA (strain R36APΔ*lytA*) also resulted in delayed lysis relative to that of the strain carrying both the phage and bacterial lysins. In conclusion, LytA activation after phage induction is not merely an inconsequential parallel process but seems to be essential for efficient phage progeny release.

Although LytA acts cooperatively with phage lysin to optimize phage progeny release, we observed that dependence solely on LytA might result in impaired phage fitness. In fact, host autolysin-induced lytic phenotypes showed a delay in lysis timing and a reduction in the proportion of total lysis. This corresponded to a delay in the release of phage particles and a significant reduction in the overall phage yield relative to what happens in the presence of both autolysin and endolysin. While retained in host cells, fully assembled phages lose the opportunity to infect naïve hosts, with a detrimental effect on phage propagation. Indeed, previous reports have shown an intimate relationship between lysis timing and phage fitness [44,46]. In addition, as the holins permeabilize the cells before lysis occurs, the LytA-induced delay in lysis traps new phages inside an already dead cell without biosynthetic capacity and thus incapable of further particle assembly. This LytA-mediated suboptimal phage release provides a provocative explanation for the crucial role of endolysins.

Another important conclusion from our data is that holin-induced lesions of the membrane not only activate phage endolysin, but also result in LytA activation. Thus, relying

simply on holin function, phages elegantly accomplish the activation of the entire lytic arsenal at their disposal. Although holin-induced LytA activation could somehow be predictable, this is a significant finding, as dissipation of the membrane proton motive force does not always trigger the autolysins. Indeed, in *B. subtilis*, the major autolysin does not respond to proton motive force-dissipating factors, despite other such enzymes being responsible for cell lysis following death from energy poisons [47-49]. Thus, our data raise the possibility that the energy status of the membrane is important in LytA regulation. However, the underlying signalling mechanism induced by depolarization to trigger the activity of LytA is not understood. Cell depolarization may induce structural and spatial changes in the membrane [17,50,51] leading to LytA activation, probably by altering the inhibitory interactions between LytA and cell wall components, such as lipoteichoic acids [16,52]. It must be emphasized that LytA activity is not always indicative of bacterial lysis, since the enzyme has been implicated in other physiological processes, such as peptidoglycan synthesis and turnover and daughter cell separation [15,53]. Given its potentially lethal activity, however, LytA is tightly regulated to ensure the maintenance of cell integrity. We may therefore speculate that these physiological functions of LytA involve small and controlled local changes in membrane architecture activating LytA in a controllable fashion. In contrast, extensive depolarization, such as that imposed by holins, with major changes in the cell membrane and consequently in the cell wall architecture, may lead to massive and uncontrolled LytA activation, resulting in cell lysis. In fact, LytA-induced lysis upon the addition of β-lactams is related to the inhibition of peptidoglycan synthesis [54], which could also induce major changes in cell wall structure.

Since LytA resides in the cell wall, our observation that LytA is activated by holin lesions leads us to speculate on an alternative regulatory mechanism of phage endolysins. Pneumococcal phage lysins are structurally and functionally similar to the bacterial cell wall hydrolase LytA [1,40-42]. Indeed, the constitutive expression of the pneumococcal phage lysin Hbl or Cpl-1 in *S. pneumoniae* M31, a mutant with the *lytA* gene deleted, restored the ability of the strain to undergo lysis in stationary phase and after exposure to DOC, two cellular responses that are dependent upon LytA activity [43]. In spite of the absence of a canonical N-terminal sequence signal, LytA is translocated across the cytoplasmic membrane [16]. Thus, pneumococcal phage lysins could also be transported by the same unknown pathway that targets LytA to the cell wall and could be subjected to the same type of physiological control. Although the canonical model of holin-lysin systems indicates that holins provide access of cytoplasmic lysins to the cell wall through holes generated in the cytoplasmic membrane, in pneumococci, holins could function simply to activate these secreted endolysins through membrane depolarization, similarly to LytA activation, rather than allowing their egress.

Taken together, our data provide the first evidence of the involvement of bacterial lysins in the progeny release of endolysin-equipped phages. Pneumococcal phage dependency on the host autolysin for optimal progeny release underscores the complex relationship between lysogenic phages and their bacterial hosts.

6. ACKNOWLEDGEMENTS

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7. SUPPLEMENTARY DATA

The following supplementary data are available for this chapter:

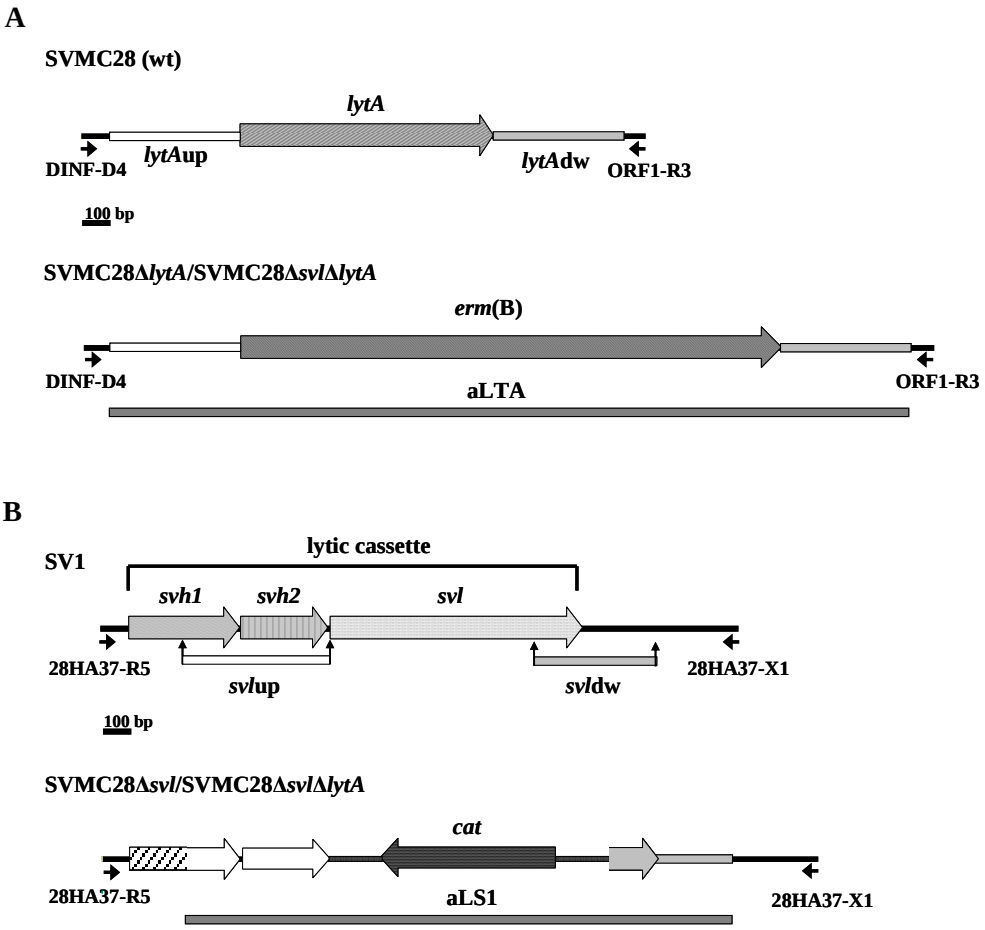

Figure II.S1. Construction of *S. pneumoniae* mutant strains in *lytA* and *svl* genes by insertion-deletion. (**A**) Organization of the *lytA* genetic region of *S. pneumoniae* in SVMC28 and mutant strains SVMC28Δ*lytA* and SVMC28Δ*svl*Δ*lytA*. The upstream and downstream regions of *lytA* (white and bright grey, respectively) were used to construct the amplicon aLTA, represented in dark grey (Table II.1). Transformation with aLTA, containing the *erm*(B) gene, results in the deletion of *lytA* gene by resistance marker replacement. This was confirmed by PCR with the indicated primers (arrows below the maps, Table II.2). (**B**) Genetic organization of the lytic cassette of phage SV1 in SVMC28 and mutant strains SVMC28Δ*svl* and SVMC28Δ*svl*Δ*lytA*. The upstream and downstream regions of *svl*, used to construct aLS1 amplicon (dark grey, Table II.1), are also shown in white and bright grey, respectively. Deletion of *svl* gene resulted from transformation with aLS1 amplicon containing the *cat* gene. This was confirmed by PCR with the indicated primers (arrows below the maps, Table II.2). Pattern arrows indicate the orientation of transcription.

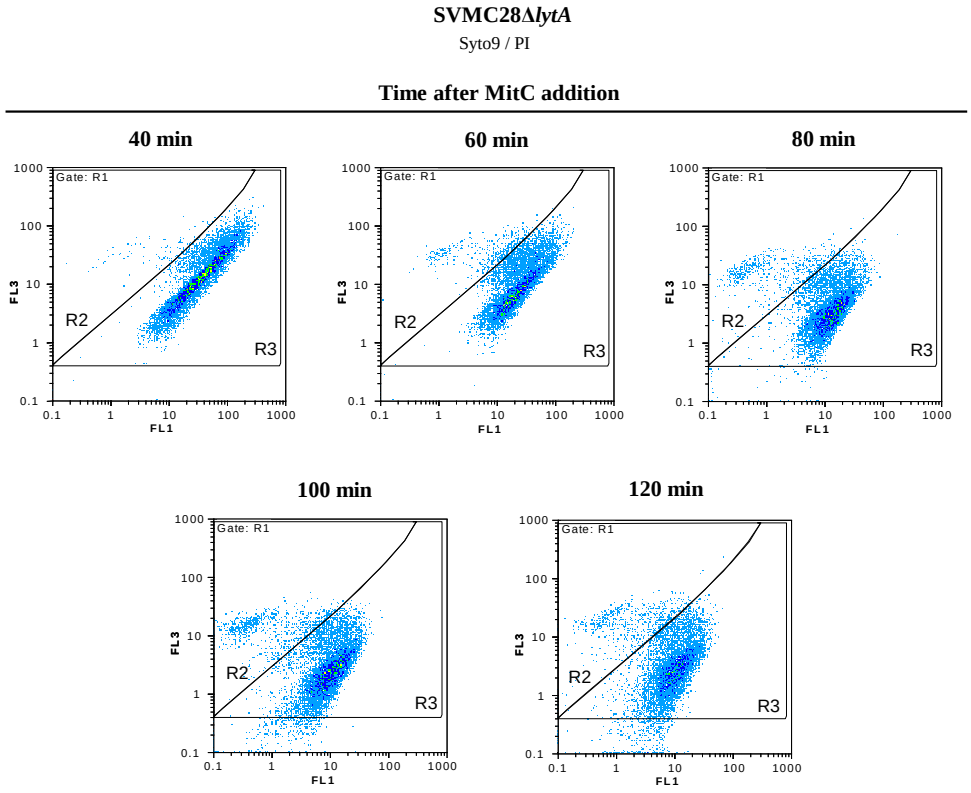

Figure II.S2. Effect of phage holin activity on SVMC28Δ*lytA* cell membrane permeabilization. Strain SVMC28Δ*lytA*, which lacks the *S. pneumoniae* autolysin LytA, was treated with MitC and tested for membrane permeabilization at various times by flow cytometry analysis using a mixture of Syto 9 and PI staining. Experimentally defined gates R2 and R3 were used to differentiate between damaged and undamaged cell populations and were designed over gate R1, which included the total stained population. In the absence of LytA, only a few damaged cells were detected, similarly to the wild-type. The data are from a representative experiment of a minimum of three independent experiments.

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CHAPTER III

HOLIN-INDEPENDENT EXPORT OF *STREPTOCOCCUS PNEUMONIAE* BACTERIOPHAGE LYSINS

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1. SUMMARY

Streptococcus pneumoniae bacteriophages (phages) have typical holin-lysin cassettes to accomplish host lysis. The widespread holin-lysin system generally relies on the holin to disrupt the cytoplasmic membrane allowing access of the endolysin to the peptidoglycan. Such strategy is assumed to operate in pneumococcus based on the extended membrane permeabilization by holins and the lack of lysin export signals. We investigated the precise lytic mechanism of pneumococcal phages by constructing lysogens without holin activity.

Upon holin deficient phage induction, lysin was produced and continuously targeted to the cell wall, acting as an exolysin. In spite of lacking a signal sequence, our data suggest that its export involves the host Sec pathway. At the bacterial surface, the phage lysin remains bound to choline in an inactive form, but is readily activated by the collapse of the cytoplasmic membrane electrochemical gradient achieved by the holins. In addition, activation of the externalized bacterial autolysin LytA, which also participates in phage-mediated lysis, is equally related to perturbations of the membrane proton motive force.

We conclude that in pneumococcal phages the lysin reaches the peptidoglycan through a holin-independent pathway and that holin-triggered lesions on the membrane activate phage and bacterial exolysins controlling lysis timing.

2. INTRODUCTION

The holin-lysin system is the main strategy adopted by bacteriophages to achieve lysis of their bacterial hosts at the end of the vegetative cycle in order to release their progeny [1]. The phage lysin is a protein characterized by having peptidoglycan degrading activity [1]. On the other hand, holins are proteins with transmembrane domains that cause lesions in the host cytoplasmic membrane [2]. According to the phage λ model for the holin-lysin system, during the infective cycle, enzymatically active endolysin accumulates in the cytoplasm, as it lacks an intrinsic secretory signal sequence, and holin accumulates in the cytoplasmic membrane without compromising its integrity [1]. At a defined time, holins disrupt the membrane allowing endolysins to gain access to the peptidoglycan target. Therefore, holins regulate lysin function hence determining the precise timing of bacterial lysis [2].

Although the holin-lysin paradigm was long though universal recent evidence questioning the absolute holin requirement for lysin export to the cell envelope has emerged. It was first reported that the Lys44 lysin from oenococcal temperate phage fOg44 carries a typical cleavable N-terminal signal sequence [3]. Lys44 is translocated to the extracytoplasmic environment by the host Sec machinery involving proteolytic removal of its signal peptide [3]. Later it was demonstrated that the *Escherichia coli* phages P1 and 21 lysins have an atypical signal sequence (SAR, signal-arrest-release) in the N-terminal domain that mediates the translocation of the enzyme without cleavage [4-6]. These lysins are exported by the host Sec system and accumulate in the periplasm as enzymatically inactive proteins anchored to the membrane by their N-terminal SAR. Surprisingly, those phages with secretory lysins also encode a holin that depolarizes the cell membrane releasing the SAR domain and thus generating the active, soluble form of the enzyme in the periplasm [4-8]. In the case of Lys44, subsequent studies revealed that lysin activity requires a sudden dissipation of the proton motive force (pmf), an event undertaken by the fOg44 holin [9]. A role for the energized membrane in regulating lysin activity was suggested [9], resembling the *E. coli* phages P1 and 21. It has been recognized that other phage exolysins, mostly of Gram-negative hosts, possess secretion signals and, therefore, are likely to be exported also independently of holin activity [1,10-12].

In *Streptococcus pneumoniae*, all phages studied so far have typical holin-lysin cassettes in their genomes [13-17]. We recently observed that, in addition to the phage lysin, holins trigger the cell wall hydrolase LytA, the major pneumococcal autolysin, with a significant contribution to phage progeny release [18]. Even though the regulatory mechanism of LytA is not understood, the activation by holins indicates that compromising membrane integrity is

sufficient for activation of LytA, which remains inactive in the cell wall, presumably attached to the choline residues of the cell membrane-linked lipoteichoic acids (LTAs) [19-23].

Sequence comparison and functional studies revealed that pneumococcal phage lysins are extremely similar to LytA [13,24-27]. Nucleotide sequences share as much as 87% identity, as demonstrated for the Hbl lysin of the HB-3 phage [27]. Additionally, both phage and bacterial lysins exhibit a bimodular structure with an N-terminal catalytic domain and a C-terminal choline binding domain [13,24]. Module shuffling between these lysins of different origins was demonstrated to result in chimeric proteins that maintain the enzymatic function [13,28]. Moreover, constitutive expression of pneumococcal phage lysins Hbl or Cpl-1 in *S. pneumoniae* strain M31 (lacking LytA), restored the ability of this strain to undergo lysis in stationary phase and after exposure to deoxycholate (DOC), two cellular responses that are dependent on LytA activity [26]. Thus, *S. pneumoniae* phage lysins may share with LytA the same cellular localization and physiological control mechanisms.

The increasingly recognition of phages with holin-independent export mechanisms of the phage lysin, together with the previously reported cell wall localization of LytA [19-23] and our observation that holins trigger LytA activity [18], led us to hypothesize that pneumococcal phage lysins, although lacking known secretion signals [13], could be targeted to the cell wall without requiring holins. In this case, holins would function to activate the already secreted lysins, in contrast to the canonical lysin-holin system believed to function in *S. pneumoniae* phages [13,15,16,29].

To test this, we investigated the cellular localization and targeting of the pneumococcal phage lysin in the absence of holin function. The activation of the lysin was also tested using compounds that mimic the permeabilizing and pmf-dissipating membrane effects of holins. We found that the phage lysin does not accumulate in the cytoplasm but is continuously exported to the cell wall during phage assembly. Despite lacking a recognizable leader peptide or other known Gram-positive cell wall targeting motifs, our data point to the involvement of the host Sec pathway in lysin export. These findings show that in *S. pneumoniae* the transport of phage lysin to the cell wall compartment is not holin-mediated and that holin function results in the activation of both the secreted phage lysin and the bacterial autolysin by membrane pmf disruption.

3. MATERIAL AND METHODS

Bacterial strains, growth conditions and sodium azide sensitivity assay

Streptococcus pneumoniae strains used in this study (Table III.1) are derivatives of strain SVMC28 (capsular type 23F, sequence type 36), which is a clinical isolate lysogenic for phage SV1 obtained from the Rockefeller University collection (A. Tomasz). All *S. pneumoniae* strains were grown in a casein-based semisynthetic medium (C+Y) at 37ºC without aeration [30] or in tryptic soy agar (TSA) (Oxoid, Hampshire, England) supplemented with 5% sterile sheep blood incubated at 37ºC in a 5% CO_2 atmosphere. Pneumococcal mutant strains were grown in the presence of 200 μg/ml kanamycin (Kn), 100 μg/ml streptomycin (Sm), 4 μg/ml chloramphenicol (Cm) and/or 2 μg/ml erythromycin (Ery) (Sigma, Steinheim, Germany), as appropriate.

To evaluate the sensitivity of the wild-type and Δ*hol*Δ*lytA* strains to sodium azide (NaN_3), cultures were grown to an optical density at 600 nm (OD_{600nm}) of 0.2 (approximately 10^8 CFU/ml) and appropriate dilutions were plated on blood-supplemented TSA plates containing 5 mM NaN_3 followed by incubation for 24 h and 48 h.

DNA manipulations

All routine DNA manipulations were performed according to standard methods [31]. PCR primers are listed in Table III.1. Chromosomal DNA from *S. pneumoniae* and phage DNA were isolated similarly to already described procedures [32,33]. PCR reactions for the purpose of constructing mutant strains were carried out with High fidelity PCR enzyme Mix kit (MBI Fermentas, Vilnius, Lithuania). PCR products were purified using the High Pure PCR product purification system (Roche, Mannheim, Germany). All oligonucleotides were obtained from Invitrogen Co. (Paisley, Scotland). Nucleotide sequences were analyzed using VECTOR NTI Deluxe (Invitrogen, Barcelona, Spain) software.

Genetic constructions

Prior to the construction of mutant strains, *S. pneumoniae* wild-type strain SVMC28 was tested for growth in the presence of Kn and Sm by seeding in TSA supplemented with the antibiotics at the appropriate concentration (200 μg/ml and 100 μg/ml, respectively). The Kn sensitivity and Sm resistance background in SVMC28 allowed the usage of janus (*kan-rpsL*$^+$) cassette [34], in a two-step transformation procedure for holin elimination.

First, strains harboring a substitution of the *hol* genes (*svh1* and *svh2*) by the *kan-rpsL*$^+$ cassette were constructed following the procedure previously described [34]. A PCR fragment containing the region immediately upstream of *svh1* was amplified from SV1 phage DNA with primers AF_H and AR_H_C, and the *kan-rpsL*$^+$ cassette was amplified from a PCR fragment containing the cassette (kindly provided by D. Morrison) with the Kan5 and DAM351 primers (Table III.1). Following purification, the two fragments were mixed and connected to generate fragment A by PCR with primers AF_H and DAM351. A fragment downstream of *svh2* gene (containing just 11 bp of *svh2*) was then amplified from SV1 DNA with the BF_H_C/BR_H primer pair (Table III.1), purified and mixed with fragment A for assembly into a unique product through PCR amplification with primers AF_H and BR_H. Strains SVMC28Δ*hol*::*kan-rpsL*$^+$ and SVMC28Δ*hol*::*kan-rpsL*$^+$Δ*lytA* were obtained following transformation of strains SVMC28 and SVMC28Δ*lytA* respectively, with the final purified PCR product by selection for Kn^r transformants (Table III.1).

Next, the *kan-rpsL*$^+$ cassette was deleted as follows. The PCR fragment containing the region immediately upstream of *svh1* was amplified with primers AF_H and AR_H_C2 and the fragment downstream of *svh2* (with 11 bp of *svh2*) was amplified with primers BF_H and BR_H. Fragments assembly into a unique product was achieved with the AF_H and BR_H primer pair (Table III.1). Strains SVMC28Δ*hol* and SVMC28Δ*hol*Δ*lytA* (without the entire cassette) were obtained following transformation of strains SVMC28Δ*hol*::*kan-rpsL*$^+$ and SVMC28Δ*hol*::*kan-rpsL*$^+$Δ*lytA* respectively, with the final purified PCR product generating Kn^s Sm^r transformants selected with Sm (Table III.1). All strains harboring a deletion in *lytA*, were also selected for chloramphenicol resistance. Besides drug selection, mutants resulting from each transformation were further confirmed by PCR with primers AF_H and BR_H. Subsequent DNA sequencing confirmed holins deletion and the integrity of phage lysin *svl* gene.

An identical strategy was used to construct the mutant strain SVMC28Δ*hol*Δ*svl* (Table III.1). The PCR fragment containing the region immediately upstream of *svh1* and the *kan-rpsL*$^+$ cassette were amplified and connected as before (fragment A). A fragment downstream of *svl* gene (containing 185 bp of *svl*) was amplified from SV1 DNA with the 2BFSVL_C/2BR_SVL primer pair (Table III.1), and assembled with fragment A with primers AF_H and 2BR_SVL. Transformation of SVMC28 with the final PCR product and selection for Kn^r transformants resulted in strain SVMC28Δ*hol*Δ*svl*::*kan-rpsL*$^+$. The *kan-rpsL*$^+$ cassette was then deleted as follows. The region immediately upstream of *svh1* and the region downstream of *svl* (with 185 bp of *svl*) were amplified with primers pairs AF_H/AR_H_C3 and 2BFSVL/2BRSVL, respectively (Table III.1). Fragments assembly into a unique product was generated with AF_H and 2BRSVL

(Table III.1). Strain SVMC28Δ*hol*Δ*svl* (without the entire cassette) was obtained following transformation of strain SVMC28Δ*hol*Δ*svl*::*kan-rpsL*$^{+}$ with the final purified PCR product by selection for Sm^{r} transformants (Table III.1). Additionally to drug selection, mutants resulting from each transformation were further confirmed by PCR with primers AF_H and 2BRSVL. Subsequent DNA sequencing confirmed holins and *svl* deletion.

Transformation of pneumococci was carried out as described [18]. Lysis upon DOC addition and after culture growth until late stationary phase confirmed the presence of LytA activity in SVMC28Δ*hol* and SVMC28Δ*hol*Δ*svl*. In strain SVMC28Δ*hol*Δ*lytA*, a nonlytic phenotype in both situations confirmed the absence of LytA activity.

Lysis assays

For induction of lysis, cultures were grown overnight at 37ºC in C+Y supplemented with the appropriate antibiotics, diluted 1:100 in fresh medium (without antibiotics) and kept at 37ºC for the rest of the incubation period. Kinetics of lysis was monitored by measuring OD_{600nm}. In experiments of induction of the phage lytic cycle, mitomycin C (MitC) (Sigma, Steinheim, Germany) was added when cultures reached approximately OD_{600nm} 0.2-0.25 to a final concentration of 0.1 μg/ml [35]. In membrane permeabilization assays, MitC-induced cultures were treated with DOC [0.04% (w/v)] (Sigma, Steinheim, Germany) at different time points. MitC-untreated controls were also treated with DOC at the same time points. In membrane pmf-dissipating assays, *N*,*N*′-dicyclohexylcarbodiimide (DCCD) (Sigma, Steinheim, Germany), an ATP synthase inhibitor [36], was added after 60 min of MitC addition at a final concentration of 100 μM. Whenever cultures were not MitC-induced, DCCD was added 60 min after the culture reached OD_{600nm} 0.2-0.25, when MitC is added in treated cultures. In Sec-dependent export assays, after 20 min of MitC induction, cultures were treated with NaN_3, a widely used SecA inhibitor, at a final concentration of 5 mM [37-40]. DCCD was added to cultures at 60 min and DOC at 200 min. As control for NaN_3 effect on viability, DCCD was not used. For cultures not treated with NaN_3, DCCD was added 60 min after MitC addition. All assays were carried out at least in duplicate.

Viability assays

Flow cytometry analysis of cultures treated with MitC and DCCD was performed. As a control for cell death, cultures were treated with DOC. Cultures were treated with MitC or DCCD as described in the lysis assays section and cells were collected after exposure at defined

intervals and then diluted in sterile-filtered 0.85% NaCl to a concentration of ~ $1x10^6$cells/ml. Cell viability was assessed by using the Live/Dead BacLight bacterial viability kit (Invitrogen, Carlbad, USA) as previously described [18]. Samples were analyzed on a Partec CyFlow space flow cytometer (Partec GmbH, Münster, Germany) with 488 nm excitation from a blue solid-state laser at 50 mW. Green fluorescence (Syto 9), indicating the population of cells without permeabilized cytoplasmic membranes, was detected in the FL1 channel and red fluorescence (PI), indicating the population of cells with permeabilized cytoplasmic membranes, was detected in the FL3 channel. Optical filters were set up such that FL1 measured at 520 nm and FL3 measured above 610 nm. The sample analysis rate was kept below 1000 events/s. Twelve thousand events were collected for each sample taken. Data were collected and analyzed by using FloMax software (Partec GmbH, Münster, Germany). Assays were carried out at least in duplicate.

Preparation of bacterial proteins

Cells were grown at an OD_{600nm} of 0.2-0.25 and then induced with MitC or left untreated. Sample aliquots (7 ml) were taken from liquid cultures at the indicated time points after MitC treatment. In the case of untreated cultures, the samples were collected at the same time points after the culture reached OD_{600nm} 0.2-0.25. Cells were harvested by centrifugation (3200 *g*, 4ºC, 10 min), washed once with 0.5 volumes of PBS 1x (PBS 10x pH 7.2, Gibco, Invitrogen, Paisley, Scotland) and ressuspended in 200 µl Tris 50 mM pH 7.5 (this cell pellet fraction was designated P). For choline wash, PBS washed cells were gently ressuspended in 200 µl choline chloride 2% (w/v) (Sigma, Steinheim, Germany) prepared in PBS 1x and incubated 30 min at 4ºC without agitation. Bacteria were collected by centrifugation (3200 *g*, 4ºC, 15 min) and the supernatant was filtered through a 0.2 µm-low-binding-protein membrane (DISMIC-03CP, Toyo Roshikaisha, Ltd., Japan) to ensure the removal of all bacteria. This choline wash fraction was designated $S_{choline}$. As control, cells were incubated in the same conditions with PBS 1x or NaCl 2% (w/v) and the wash fractions were designated S_{PBS} and S_{NaCl}, respectively. The pellet was then washed once with 0.5 volumes of PBS 1x and ressuspended in 200 µl Tris 50 mM pH 7.5. If cells were previously washed with choline, this cell pellet fraction was called $P_{choline}$; if cells were subjected to control PBS wash, the fraction was called P_{PBS}.

In Sec-dependent export assays, cells grown at an OD_{600nm} of 0.2-0.25 were induced with MitC and 20 min after induction, cultures were treated with NaN_3 at final concentration of 5 mM. Cells treated only with MitC were used as control for NaN_3 effect. As before, sample aliquots (7 ml) were taken from liquid cultures at the indicated time points after MitC

treatment. Cell pellets (P) and choline wash fraction ($S_{choline}$) were prepared as described above.

SDS-PAGE and immunoblotting

Polyclonal antibody (pAb) against pneumococcal autolysin LytA was kindly provided by P. Garcia [41], monoclonal antibody (mAb) 144,H-3 that recognizes streptococcal elongation factor Ts was provided by J. Kolberg [42] and pAb antibody to CodY was a gift of A. Sonenshein [43]. mAb against pneumococcal pneumolysin (Ply) was purchased from Statens Serum Institute (Copenhagen, Denmark). For SDS-PAGE electrophoresis, samples (5 or 10 µl of total cells and 45 µl of cell washes) were boiled for 5 min with sample buffer containing 10% β-mercaptoethanol at a final concentration of 1x. Samples were electrophoresed in 5% acrylamide stacking and 12% separating gels and proteins were electrotransferred to 0.20 µm nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Protein molecular mass marker Precision Plus Protein Standard was used (Bio-rad, California, USA). For immunodetection, anti-LytA (rabbit), anti-Ply (mouse), anti-Cody (rabbit) and 144,H-3 anti-L7/L12 and Ts (mouse) antibodies were used at a dilution of 1:10000, 1:1000, 1:10000 and 1:5000, respectively. mAb 144,H3 was used as hybridoma supernatant fluid. Secondary antibodies anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology Inc., California, USA) were diluted 1:5000. After incubation, blocking and washing procedures, blots were developed using the Pierce ECL western blotting substrate (Thermo Fisher Scientific Inc., Rockford, USA), according to the manufacturer's instructions, and exposed to Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK).

Table III.1. Bacterial strains and primers used in this study.

Strain or primer	Description[a]	Source or reference
S. pneumoniae		
SVMC28	Parental strain lysogenic for phage SV1; LytA$^+$, Svl$^+$, Svh1$^+$ and Svh2$^+$; Kn^s Sm^r	[18,25]
SVMC28Δ*hol*::*kan-rpsL*$^+$	SVMC28 but Δ*hol*::*kan-rpsL*$^+$; LytA$^+$, Svl$^+$, Svh1$^-$ and Svh2$^-$; Kn^r Sm^s	This study
SVMC28Δ*hol*	SVMC28 but Δ*hol* (*kan-rpsL*$^+$ cassette deleted); LytA$^+$, Svl$^+$, Svh1$^-$ and Svh2$^-$; Kn^s Sm^r	This study
SVMC28Δ*lytA*	SVMC28Δ*lytA*::*cat*; LytA$^-$, Svl$^+$, Svh1$^+$and Svh2$^+$; Cm^r Kn^s Sm^r; control in lysis assays	[18]
SVMC28Δ*hol*::*kan-rpsL*$^+$Δ*lytA*	SVMC28Δ*lytA* but Δ*hol*::*kan-rpsL*$^+$; LytA$^-$, Svl$^+$, Svh1$^-$ and Svh2$^-$; Cm^r Kn^r Sm^s	This study
SVMC28Δ*hol*Δ*lytA*	SVMC28Δ*lytA* but Δ*hol* (*kan-rpsL*$^+$ cassette deleted); LytA$^-$, Svl$^+$, Svh1$^-$ and Svh2$^-$; Cm^r Kn^s Sm^r	This study
SVMC28Δ*hol*Δ*svl*::*kan-rpsL*$^+$	SVMC28 but Δ*hol*Δ*svl*::*kan-rpsL*$^+$; LytA$^+$, Svl$^-$, Svh1$^-$ and Svh2$^-$; Kn^r Sm^s	This study
SVMC28Δ*hol*Δ*svl*	SVMC28 but Δ*hol*Δ*svl* (*kan-rpsL*$^+$ cassette deleted); LytA$^+$, Svl$^-$, Svh1$^-$ and Svh2$^-$; Kn^s Sm^r	This study
SVMC28Δ*svl*	SVMC28 Δ*svl*::*cat*; LytA$^+$, Svl$^-$, Svh1$^+$ and Svh2$^+$; Cm^r; control in lysis assays	[18]
SVMC28Δ*svl*Δ*lytA*	SVMC28 Δ*svl*::*cat* Δ*lytA*::*erm*(B) ; LytA$^-$, Svl$^-$, Svh1$^+$ and Svh2$^+$; Cm^r Ery^r; control in viability assays	[18]
CP1500	Nov^r; donor of point markers, control in transformation assays	[44]
Primers[b]		
AF_H	CTTAACCAAGCCTTACGAATGAC; upstream of *svh1*; FJ765451	This study
AR_H_C	CATTATCCATTAAAAATCAAACGGCTCCTATTCTTTAGGTTCTCCCG (underlined sequence corresponding to *kan* cassette; complementary to primer Kan5); upstream of s*vh1* and downstream of AF_H; FJ765451	This study
Kan5	CCGTTTGATTTTTAATGGATAATG	[34]
DAM351	CTAGGGCCCCTTTCCTTATGCTTTTGGAC	[34]
BF_H_C	GCATAAGGAAAGGGGGCCTAGGGAAAGGACGATAGGGAATGG (underlined sequence corresponding to *rpsL*; complementary to primer DAM351); downstream of *svh2*; FJ765451	This study
BR_H	CTTGCTTAAACTGTTCACGGC; downstream of *svh2* and downstream of BF_H_C; FJ765451	This study
AR_H_C2	CCATTCCCTATCGTCCTTTCCCTCCTATTCTTTAGGTTCTCCCG (underlined sequence corresponding to region downstream of *svh2*; complementary to primer BF_H); upstream of *svh1* and downstream of AF_H; FJ765451	This study
BF_H	GGAAAGGACGATAGGGAATGG; downstream of *svh2* and upstream of BR_H; FJ765451	This study
2BFSVL_C	GCATAAGGAAAGGGGCCCTAGTGAAGACAGGCTGGGTCAAGTAC (underlined sequence corresponding to *rpsL*; complementary to primer DAM351); downstream of *svl*; FJ765451	This study
2BRSVL	GCTATTTCCCAAGGTGCTGG; downstream of *svl* and downstream of 2BFSVL_C; FJ765451	This study
AR_H_C3	GTACTTGACCCAGCCTGTCTTCACTCCTATTCTTTAGGTTCTCCCG (underlined sequence corresponding to region downstream of *svl*; complementary to primer 2BFSVL); upstream of *svh1* and downstream of AF_H; FJ765451	This study
2BFSVL	TGAAGACAGGCTGGGTCAAGTAC; downstream of *svl* and upstream of 2BRSVL; FJ765451	This study

[a] Kn^r, kanamycin resistance; Sm^r, streptomycin resistance; Cm^r, chloramphenicol resistance; Ery^r, erythromycin resistance; Nov^r, novobiocin resistance.
[b] Information given in the order sequence; gene; and SV1 lytic cassette GenBank accession number.

4. RESULTS

Phage lysin and bacterial autolysin are not able to cause phage-mediated lysis in the absence of holin function

In order to study how the phage holin-lysin mechanism operates in *S. pneumoniae*, we constructed holin-deficient Δ*hol* lysogens. We reasoned that exploring both the cellular localization of the phage lysin and its triggering signals in a context without holin activity would offer insights into how holin and lysin functions integrate to achieve bacterial host lysis.

The holin mutants were generated in pneumococcal strain SVMC28, lysogenic for phage SV1 (Table III.1). The lytic cassette of SV1 was previously characterized (Genbank accession number FJ65451) and contains two genes, *svh1* and *svh2*, just upstream of the *svl* gene encoding the lysin Svl [18], which potentially encode two small proteins of 140 and 110 amino acids with a predicted molecular mass of 15.8 kDa and 11.9 kDa, respectively. The putative Svh1 and Svh2 proteins show high amino acid sequence identity (81.1% to 98.6%) to the two predicted holins from the *S. pneumoniae* lysogenic phages MM1 [17], VO1 [45] and 23782 [46]. Analysis of the predicted structure of the potential Svh holins using SOSUI (http://www.expasy.org) also reveals three and one potential hydrophobic transmembrane regions for Svh1 and Svh2 (with a N-out, C-in topology), a characteristic of class I and III holins [2,47], respectively. Moreover, as previously reported, attempts to clone *svh1* and *svh2* in *E. coli* resulted in loss of viability [18]. Therefore, the characteristics of the putative Svh proteins suggest that these proteins correspond to the holins of phage SV1.

To eliminate holin activity, both *svh1* and *svh2* genes were deleted. All mutant strains displayed growth rates similar to the wild-type strain regardless of genetic manipulations. As expected, *lytA*-deficient strain Δ*hol*Δ*lytA* was resistant to autolysis in stationary phase (Fig.III.1A) and when treated with DOC (Fig III.2A2), two cellular responses dependent upon LytA. In contrast, Δ*hol* and Δ*hol*Δ*svl* show lysis in stationary phase (Fig.III.1A) and in response to DOC (data not shown) as expected from a fully functional autolysin.

As holins permeabilize the pneumococcal membrane triggering the activity of both phage and bacterial lysins [14,18], the elimination of holin activity would be expected to prevent activation of both lysins. This was confirmed by inducing the SV1 lytic cycle with MitC in the Δ*hol* mutants (Δ*hol*, Δ*hol*Δ*lytA* and Δ*hol*Δ*svl*). As anticipated, in the absence of holins, phage induction and a full course of phage infectious cycle did not culminate in host lysis at the expected time, despite the presence of LytA and Svl (Fig.III.1A). In fact, no lysis was observed in the Δ*hol* mutants even two hours after its onset in the holin-carrying strains Δ*svl* and Δ*lytA*, a

time when lysis already reached its full extent in these strains with functional holins (Fig.III.1A). Cultures eventually lysed but only after a long MitC exposure, which is possibly related to the autolytic process occurring in late stationary phase [48] exacerbated by the accumulation of intracellular phages and phage proteins that have been shown in phage λ to continue to build up when holins fail to determine the moment of lysis [1,49].

To further verify the abolishment of holin activity we tested the membrane integrity by flow cytometry after phage induction. In this assay, cells with permeabilized membranes allow the uptake of PI, fluorescing in the FL3 channel, while cells with nonpermeabilized membranes internalize only Syto 9, fluorescing in FL1. The gates representing permeabilized (R2) and nonpermeabilized cells (R3) were constructed as previously described [18]. In both Δ*hol*Δ*svl* and Δ*hol*Δ*lytA* strains, MitC-treated cells are always found in gate R3 (Fig.III.1B), whether cells are analyzed before (40 min), at the onset (80 min) or at the end (120 min) of the lytic process observed in the Δ*svl* and Δ*lytA* strains possessing functional holins (Fig.III.1A). Indeed, at 120 min only a small fraction of the cells are found in gate R2, with approximately 96% of cells maintaining membrane integrity. Similar results were obtained for the strain lacking holin activity but retaining both lytic functions (supplementary data, Fig.III.S1). In contrast, a strain with intact holin function but Δ*svl*Δ*lytA* becomes increasingly permeabilized after phage induction and at 120 min cells were found mostly in gate R2 (Fig.III.1B), as previously reported [18]. Collectively, the absence of membrane permeabilization and lysis demonstrates the successful elimination of holin activity and confirms that holins are required to activate lysins.

Premature phage lysin-mediated lysis is induced by DOC that mimics holin function

To further explore the pneumococcal holin-lysin mechanism, we examined lysin production throughout the lytic cycle before holin action. Since phage lysins are activated through membrane permeabilization caused by holins and the permeabilizing properties of DOC were previously confirmed by flow cytometry [18], DOC addition should result in phage lysin activation similarly to LytA. DOC was then added at several time points after phage induction to the Δ*hol*Δ*lytA* strain containing a functional phage lysin. In the absence of autolysin activity, lysis promoted by DOC should be related to phage lysin concentration.

As can be seen in Fig.III.2A1, the addition of DOC to MitC-treated Δ*hol*Δ*lytA* cultures resulted in a gradual increase of lysis with time reaching a substantial percentage from 60 min onwards (over 75%). In contrast, only modest DOC-mediated lysis is observed at any time point in MitC-untreated cultures, with only 30% lysis at 60 min (Fig.III.2A2).

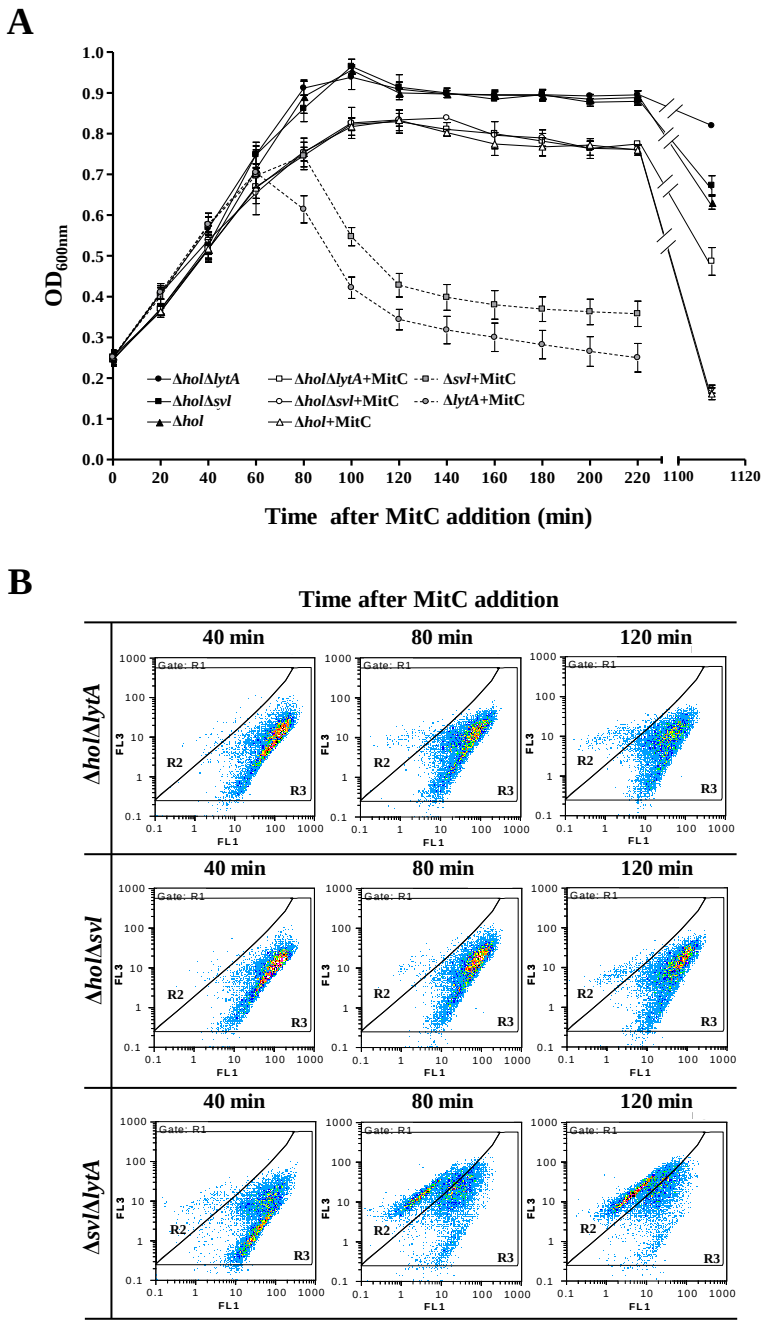


Figure III.1. Effect of holin deletion in pneumococcal phage-induced lysis. (**A**) Absence of lysis in mutants lacking holins. SVMC28 derived mutants lacking holins with or without functional LytA and Svl were grown until OD_{600nm}= 0.2-0.25 and 0.1 μg/ml of MitC was added to induce phage excision (t=0 min). As control for lysis, mutants carrying holins but deleted in LytA or Svl were also induced (dotted lines). Results are an average of a minimum of three independent experiments. Error bars represent 95% confidence intervals. (**B**) Maintenance of membrane integrity. Exponentially growing cells were treated with MitC (as indicated in panel A), collected after 40, 80 and 120 min and tested for membrane permeabilization by flow cytometry following staining with a mixture of Syto 9 and propidium iodide (PI) (Live/Dead BacLight bacterial viability kit, Invitrogen, Carlbad, USA). Similar analysis patterns were obtained for SVMC28Δ*hol* (supplementary data, Fig.III.S1). As control for holin-induced permeabilization, strain SVMC28Δ*svl*Δ*lytA* was also studied and the results were similar to those already described [18]. Gate R2 corresponds to the membrane damaged population, while gate R3 corresponds to the population with intact membrane. Both gates were designed over gate R1, which includes the total stained population. Results are representative of a minimum of two independent experiments.

Western blot was also performed with the Δ*hol*Δ*lytA* strain using an antibody anti-LytA that recognizes the phage lysin due to the high similarity between LytA and Svl (89.3% identity at the amino acid level). A band of approximately 36 kDa was detected corresponding to the predicted mass of Svl (36.53 kDa). In agreement with the observed lysis, immunoblotting analysis of the MitC-treated culture revealed a large amount of Svl at 60 min (Fig.III.2B). When the same cells were not treated with MitC, only a residual amount of Svl was detected. The considerable increase in the quantity of Svl upon MitC treatment is consistent with induction of the phage lytic cycle since MitC treatment has little influence on the synthesis of the bacterial proteins elongation factor Ts and pneumolysin Ply (Fig.III.2B). In the absence of MitC, phage induction can still occur spontaneously although at a lower rate [50,51] with the subsequent synthesis of Svl (Fig.III.2B). Accordingly, the Svl accumulated in these conditions may be responsible, possibly together with other bacterial lysins, for the residual lysis observed (Fig.III.2A2, Δ*hol*Δ*lytA* MitC$^-$; see also Fig.III.S2). Taken together these data indicate a progressive synthesis of pneumococcal phage lysin across the lytic cycle until lysis.

Comparing Δ*hol*Δ*lytA* and Δ*lytA*, which differ only in the presence of functional holins, the lysis pattern due to DOC is the same up to 60 min of MitC treatment (Fig.III.2A1 and A3). Importantly, in both strains a significant lysis percentage (observed at 60 min) occurs upon DOC treatment before the normal time of lysis defined by holin action at 80 min, demonstrating that phage lysin build up does not by itself lead to bacterial lysis. Regardless of a significant accumulation of Svl, lysis was blocked until DOC was added to permeabilize the membrane. Thus, in *S. pneumoniae* a mechanism regulating phage lysins is functioning to prevent premature bacterial lysis.

Phage lysin is cell wall localized and the targeting is not dependent upon holin

Since DOC permeabilizes the membrane lipid bilayer, the observed lysis mediated by the phage lysin could result from compromising the barrier function of the cytoplasmic membrane. Thus, the release of intracellularly accumulated lysins onto their peptidoglycan substrate cannot be excluded. We next designed experiments to determine the exact location of the phage lysin. It was shown that LytA contains a choline binding domain that attaches the protein to the cell surface namely to the choline residues in the peptidoglycan-bound teichoic acids and lipoteichoic acids [13,19,21,23]. It was also shown that a 2% choline solution could compete for the binding sites leading to the release of LytA from the bacterial surface [19,52]. Due to high functional and structural similarity between LytA and pneumococcal phage lysins, including Svl, the effect of choline on phage lysin attachment was examined. To avoid holin-

mediated phage lysin escape that could lead to misinterpretation of its localization, lysogens without holin function were used.

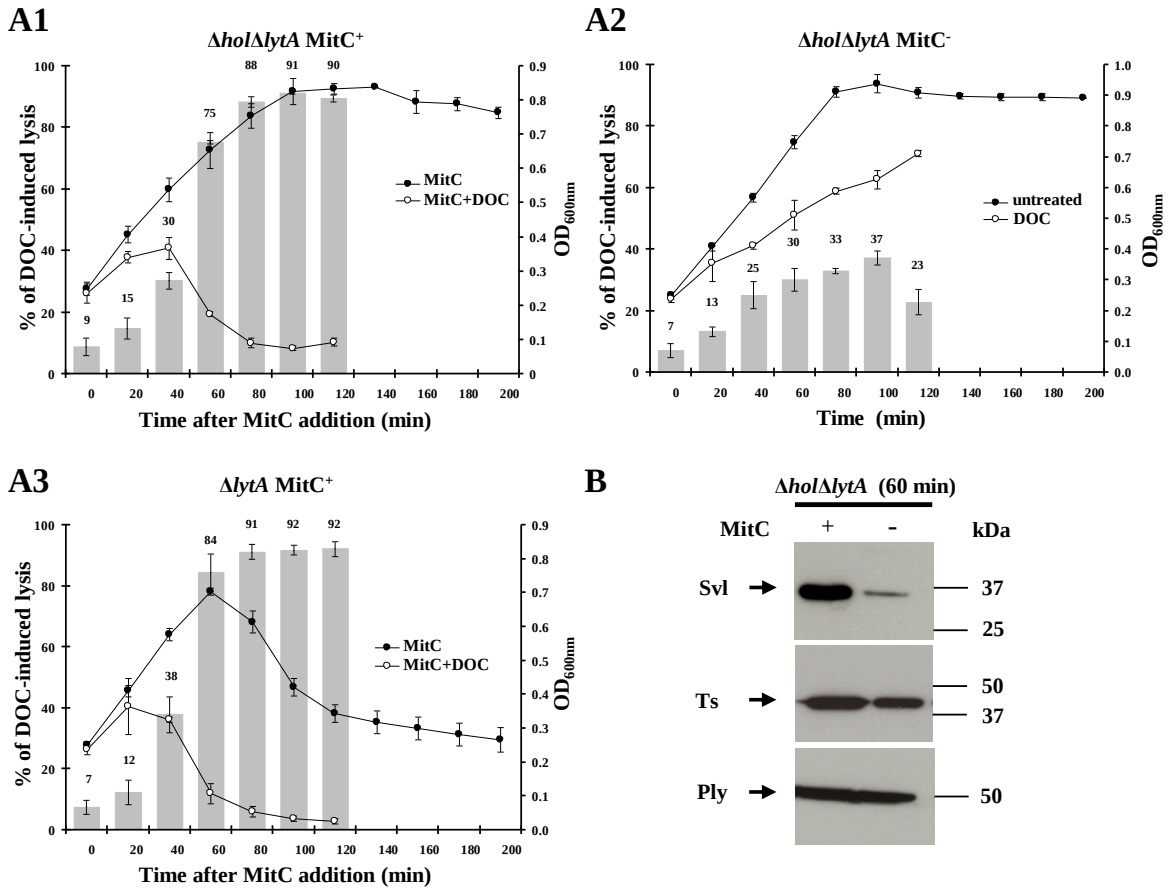


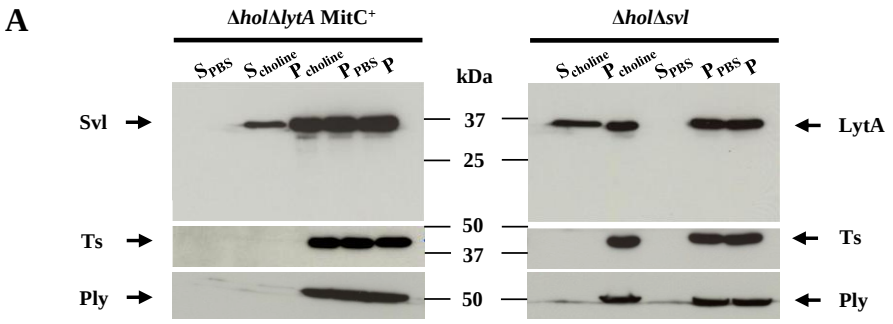
Figure III.2. Premature phage lysin-mediated lysis of *S. pneumoniae* is triggered by membrane permeabilization. (**A1, 2** and **3**) Kinetics of DOC-induced lysis after phage induction. SVMC28Δ*lytA* and SVMC28Δ*hol*Δ*lytA* strains were grown until OD_{600nm}=0.2-0.25 and 0.1 µg/ml of MitC was added to induce phage excision (t=0 min). Cultures were treated with DOC [final concentration of 0.04% (w/v)] at 20-min intervals after phage induction until 120 min. OD_{600nm} is indicated before (full symbols) and 20 min after DOC addition (open symbols) and DOC-triggered lysis is expressed as percentage of optical density drop (values are shown above the bars). As control, MitC-untreated SVMC28Δ*hol*Δ*lytA* was also treated with DOC. Results are an average of a minimum of three independent experiments. Error bars represent 95% confidence intervals. (**B**) Phage lysin synthesis and accumulation after phage induction. SVMC28Δ*hol*Δ*lytA* was treated with MitC (as indicated in panel A) or left untreated. After 60 min of MitC addition, cells were harvested by centrifugation. In the case of untreated cultures, equal amounts of cells were collected at 60 min after the culture reached OD_{600nm} 0.2-0.25 (when MitC is added to the treated cultures). Samples were processed for SDS-PAGE and western blotting to check for phage lysin Svl production with anti-LytA antibody. 10 µl of each sample was run in each lane. Imunodetection of the pneumococcal proteins Ts (cytoplasmic elongation factor) and Ply (cell wall pneumolysin) was also performed with antibodies 144,H-3 and anti-Ply, respectively. The mass of the molecular mass markers is indicated. Results are representative of a minimum of two independent experiments.

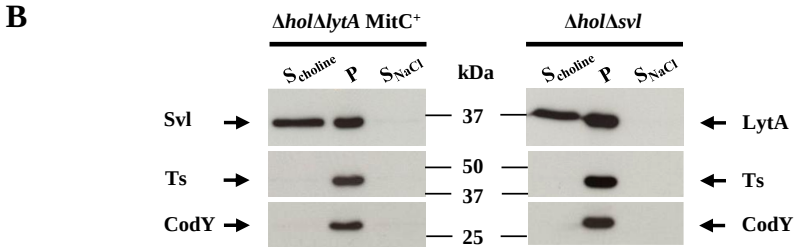
When lysogens expressing Svl (Δ*hol*Δ*lytA* MitC$^+$) were washed with 2% choline in PBS, 60 min after phage induction with MitC, Svl was found in a significant amount in the choline wash ($S_{choline}$) whereas no phage lysin was released from the cells by washing with PBS only (S_{PBS}) (Fig.III.3A) or 2% NaCl solution (S_{NaCl}) (Fig.III.3B), ruling out lysin extraction due to the high ionic strength of the choline solution. This observation suggests that the phage lysin is located in the cell wall even in the absence of the holin. As a control, the cell wall localized LytA was also extracted by the choline solution and found solely in $S_{choline}$ (strain Δ*hol*Δ*svl*, Fig.III.3A and B). Moreover, cytoplasmic Ts was not detected in the choline, PBS and NaCl washes excluding contamination of the wash fractions with cells or components of the cytoplasm due to cell lysis and also guaranteeing that Svl present in $S_{choline}$ results exclusively from the action of choline. Similar results were obtained with another known cytoplasmic protein, CodY, reported before to successfully control for cell lysis in *S. pneumoniae* [53]. Finally, Ply was used as another control for the choline wash. Ply was recently demonstrated to localize at the cell surface [53], but since it is not a choline binding protein it is not expected to be affected by choline. Although almost undetectable, Ply was present in the wash fractions ($S_{choline}$ and S_{PBS}), which is consistent with its noncovalent binding to the cell wall [53], but it is detected in equivalent amounts in both choline and PBS washes strongly supporting the specificity of the choline wash to remove only choline binding proteins such as Svl and LytA.

Overall, it can be concluded from these findings that pneumococcal phage lysin localizes to the cell surface and is dependent on interactions with choline for cell wall association. Furthermore, as in the absence of holins the phage lysin is found in the cell wall, holins are not required for phage lysin transport across the *S. pneumoniae* cytoplasmic membrane.

Choline washing was used to further explore the surface localization of phage lysin on intact cells across the lytic cycle. Δ*hol*Δ*lytA* lysogens were treated with MitC, samples of equal volume were collected at different time points and the presence of Svl was assayed in the pellet (P) and wash fractions (S). Western blot analysis of the phage lysin production during SV1 lytic cycle indicated that the protein was detectable in pelleted cells at all times (Fig.III.3C). The Svl detection when MitC is added (t=0 min) can be attributed to spontaneous events, as mentioned above. With increasing time, however, MitC phage induction leads to increasing concentrations of Svl until the moment of lysis at 80 min. To better evaluate this, cytoplasmic accumulation of Ts was also followed. Although an increase of Ts was expected, as phage induction by MitC arrests cell division but cells continue to elongate and increase in mass [25,54], we observed that this increase is not as marked as that of Svl. Svl expression at the end of the lytic cycle is 6.7-fold higher when normalized by Ts (Fig.III.3C). Analogous observations were extended to the wash fractions. Choline led to the elution of increasing

amounts of Svl with time (Fig.III.3C). This amount of Svl extracted by choline was proportional to the amount accumulated in the cells ($S_{choline}$ *vs* P, Fig.III.3C upper and lower panel). Control PBS washes did not promote Svl removal. Again, Ts cytoplasmic control revealed no detectable cell lysis or cell contamination in any of the wash samples, with Ts being detectable only in the intact bacteria. The results were clearly indicative of continuous holin-independent targeting of the phage lysin to the cell wall accompanying its synthesis.





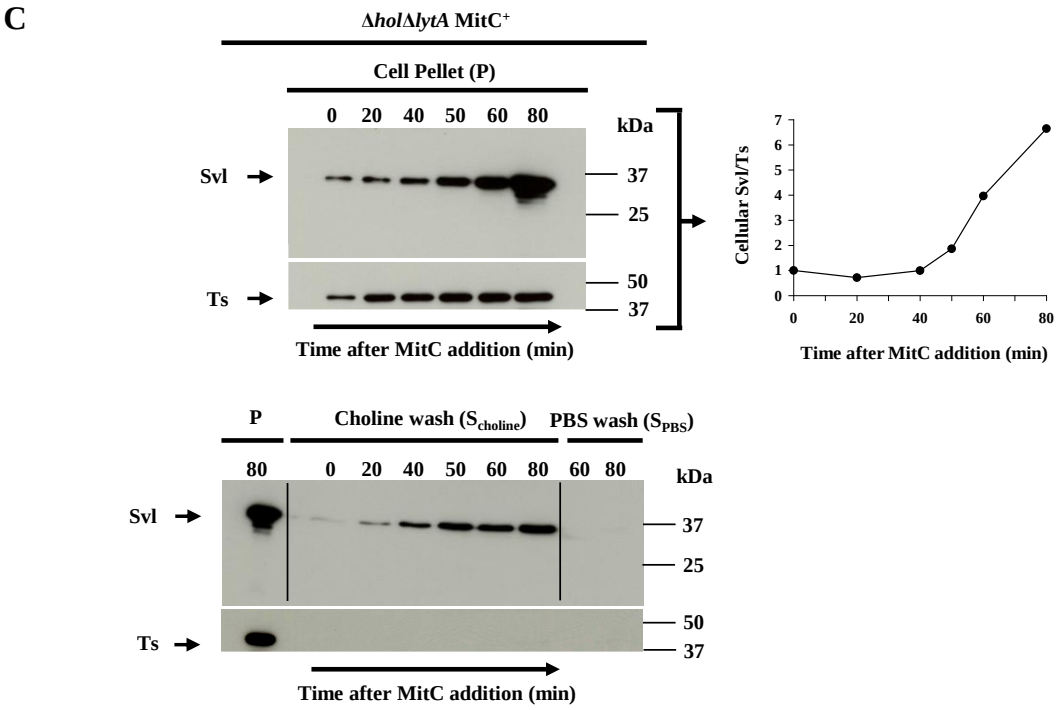


Figure III.3. Holin-independent cell wall localization of pneumococcal phage lysins. (**A** and **B**) Choline is able to extract phage lysins from intact bacteria. SVMC28Δ*hol*Δ*lytA* was treated with MitC (as indicated in Fig.III.1A) for phage induction. After 60 min of MitC addition, equal amounts of cells were harvested by centrifugation. Cells that were directly ressuspended in Tris 50 mM pH 7.5 correspond to the cell pellet fraction, P. Cells that were choline washed originated the supernatant choline wash fraction $S_{choline}$ and the cell pellet fraction $P_{choline}$. Control samples that were PBS or NaCl washed, instead of choline, originated S_{PBS} and P_{PBS} or S_{NaCl}. All fractions were tested by western blotting for Svl presence with anti-LytA antibody. LytA was used as control for choline wash, thus equivalent samples were also collected 60 min after MitC-untreated SVMC28Δ*hol*Δ*svl* culture reached OD_{600nm} 0.2-0.25. To control any contamination of fractions and the specificity of the choline washes, all fractions were tested for pneumococcal cytoplasmic elongation factor Ts (antibody 144,H-3), cytoplasmic protein CodY (antibody anti-CodY) and cell associated pneumolysin Ply (antibody anti-Ply) that is not expected to be removed by choline. (**C**) Time course of phage lysin synthesis and cell wall targeting. Equal aliquots were taken from MitC-treated SVMC28Δ*hol*Δ*lytA* culture at 0, 20, 40, 50, 60 and 80 min. Cells were harvested by centrifugation and directly ressuspended in Tris 50 mM pH 7.5 (cell pellet fraction, P) or choline washed to remove choline binding proteins (choline wash fraction, $S_{choline}$). As control, cells collected at 60 and 80 min were also washed with PBS only (S_{PBS}). All fractions were tested by western blotting for Svl presence with anti-LytA antibody. P and S fractions were also tested for Ts (antibody 144,H-3) to control for the accumulation of cytoplasmic proteins throughout time and in the choline wash fraction to control for cytoplasmic contamination. The ratio between Svl and Ts expression in pelleted cells was determined and the Svl increase with time is represented. 10 µl (panel A) or 5 µl (panels B and C) of each P fraction and 45 µl of each S fraction were run in each lane. The mass of the molecular mass markers is indicated. Results are representative of a minimum of two independent experiments.

Membrane pmf dissipation is essential and sufficient for triggering phage lysin and bacterial autolysin

From the above results pneumococcal phage exolysins do not require the hole-forming capacity of holins for their delivery to the peptidoglycan. However, our data point to a strict holin requirement for lysin activity (this study, [18]). In this case, holins could function to collapse the cytoplasmic membrane electrochemical gradient, leading to the activation of the externalized lysin, in a similar fashion to the holins of exolysin-carrying phages [5,6,9,55].

To test this hypothesis, we used the membrane pmf-dissipating agent *N*,*N*´-dicyclohexylcarbodiimide (DCCD) that inhibits the ATP synthase depleting the proton gradient and thus, leading to the disruption of the pmf [36]. As shown in Fig.III.4A, addition of DCCD to Δ*hol*Δ*lytA* cultures 60 min after the phage induction with MitC triggered complete host lysis. In contrast, negligible lysis was observed with DCCD in the absence of phage lysin (Δ*hol*Δ*lytA* without MitC treatment, Fig.III.4A). These results indicate that, in the absence of holins, DCCD activates the phage lysin resulting in cell lysis. Likewise, incubation of the lysogen Δ*hol*Δ*svl* (expressing LytA) with DCCD always triggered lysis independently of MitC treatment (Fig.III.4A). Thus, DCCD-induced pmf dissipation also activates the bacterial LytA, known to be holin-activated in phage release (this study, [18]).

To confirm that the observed lysis was not the result of cytoplasmic membrane damage that allowed the phage lysin to reach the peptidoglycan, we analyzed the effect of DCCD on membrane permeabilization by flow cytometry. Strain Δ*hol*Δ*lytA* without MitC treatment was

used to avoid lysis due to the presence of the autolysin or the phage lysin. As before, cells with permeabilized membranes allow the uptake of PI and distribute within gate R2 whereas cells with intact membranes that internalize only Syto 9 are found in gate R3. The Δ*hol*Δ*lytA* cells collected at the moment of DCCD addition (t=0 min, corresponding to 60 min in the growth curve in panel A) are almost exclusively contained in R3, like the untreated control (Fig.III.4B). This demonstrates that exposure to DCCD does not instantaneously permeabilize membranes in agreement with DCCD's mode of action, that dissipates the pmf but does not create membrane lesions. This is in contrast to DOC that has an immediate permeabilizing effect, as can be seen by the two well-defined populations concentrated evenly in both gates when a 50% mixture of DOC and DCCD-treated cells (at t=0 min) was analyzed (Fig.III.4B). Even after 140 min of DCCD exposure (corresponding to 200 min in the growth curve in panel A, when lysis is practically complete if either LytA or Svl are present), the distribution pattern is very distinctive from that of DOC-treated cells, with very few cells in gate R2. It is highly unlikely that this low percentage of cells that internalized PI and thus, may have permeabilized membranes, resulted in the extended lysis observed upon DCCD treatment of strains with functional lysins (see for instance strain Δ*hol*Δ*lytA*+MitC+DCCD, Fig.III.4A), considering that in lysis promoted by holins almost all Δ*svl*Δ*lytA* cells were found to be permeabilized by flow cytometry (Fig.III.1B, [18]). We conjecture that this small degree of permeabilization may be a consequence of an extended time under the effect of DCCD.

Taken together, these data indicate that the pneumococcal phage lysin is already positioned in the cell wall at lysis onset as collapse of the membrane pmf cannot allow protein passage. We conclude that pneumococcal phage lysin is externalized by a holin-independent mechanism and holin-mediated membrane pmf dissipation is required and sufficient for its activation.

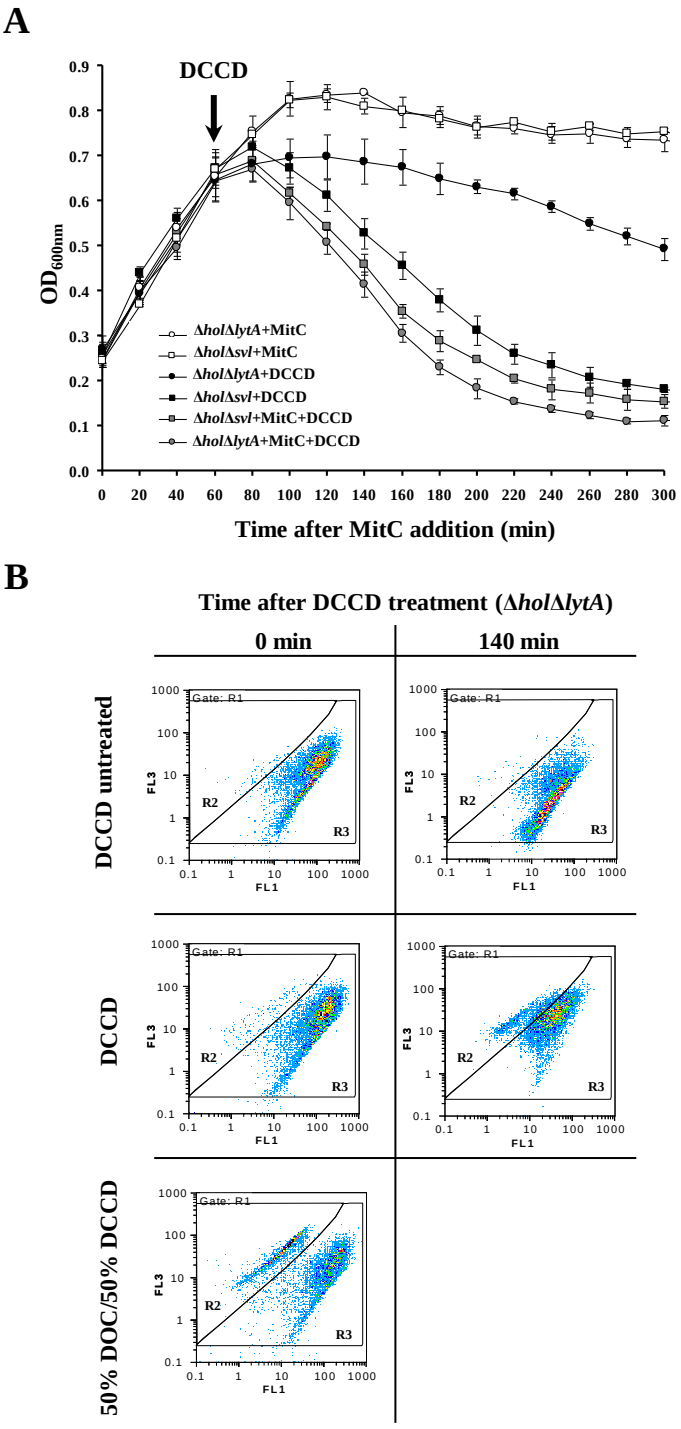


Figure III.4. Pneumococcal lysis mediated by phage lysin and bacterial autolysin LytA is triggered by membrane pmf dissipation. (**A**) Kinetics of DCCD-induced lysis with and without phage induction. SVMC28 derived mutants lacking holins and LytA or Svl were grown until OD_{600nm}=0.2-0.25 and 0.1 μg/ml of MitC was added to induce phage excision (t=0 min). After 60 min of MitC addition, the pmf-dissipating agent DCCD (100 μM) was added to the cultures (indicated by arrow). When cultures were not MitC-induced, DCCD was added at 60 min after the cultures reached OD_{600nm} 0.2-0.25 (when MitC is added to the treated cultures). As control for DCCD-induced lysis, cultures were induced with MitC but not treated with DCCD. Results are an average of a minimum of three independent experiments. Error bars represent 95% confidence intervals. (**B**) Minimal membrane permeabilization effect of pmf-dissipating agent DCCD. SVMC28Δ*hol*Δ*lytA* cells were treated with DCCD 60 min after the cultures reached OD_{600nm} 0.2-0.25 (when MitC is added in other cultures). Cells were collected immediately after DCCD addition (t=0 min) and 140 min after treatment, corresponding to 60 min and 200 min in the curves shown in panel A. Also a fraction of the same culture was left untreated. Again, t=0 min and t=140 min in the flow cytometry analysis corresponds to 60 min and 200 min after OD_{600nm} 0.2-0.25, respectively. As control for massive membrane permeabilization, cells were also treated with DOC [0.04% (w/v)] 60 min after OD_{600nm} 0.2-0.25. Cells were stained with a mixture of Syto 9 and propidium iodide and analyzed on a flow cytometer. For definition of gates R2 and R3 see Fig.III.1. Results are representative of a minimum of two independent experiments.

Involvement of the Sec pathway in phage lysin targeting to the cell wall

Phage lysin cell wall localization implies its translocation across the membrane. It is known that the Sec export pathway is highly conserved among bacteria [56], which led us to investigate if this host system was involved in the extracytoplasmic targeting of *S. pneumoniae* phage lysins. For that, we utilized sodium azide (NaN_3), a widely used inhibitor of the SecA ATPase activity that was established to suppress protein translocation by the Sec pathway in both Gram-negative and Gram-positive bacteria [39,40] including *Streptococcus gordonii* [38] and *Streptococcus parasanguinis* [37], both closest related to *S. pneumoniae*. To Δ*hol*Δ*lytA* cultures, NaN_3 was added (at a 5 mM concentration that allows cell growth) shortly after MitC induction of Svl expression. We then tested the effect of DCCD since it can only trigger lysis in strains with externalized lysins (see above).

The addition of DCCD to MitC and NaN_3-treated cultures did not result in cell lysis contrary to cultures treated only with MitC where DCCD activated the phage lysin (Fig.III.5A). However, when DOC is added to NaN_3-treated cultures to permeabilize the membrane lysis occurred, indicating that phage lysins were synthesized and accumulated in the cells. This indicates that, in the absence of holin, NaN_3 blocks the membrane translocation of the phage lysin. To further support this, we examined the NaN_3 effect on the capacity of choline to extract Svl from the cell surface. SDS-PAGE analysis at selected time points of the choline washes of MitC-induced Δ*hol*Δ*lytA* cells treated with NaN_3 showed that from 30 min onwards of NaN_3 exposure (50 min after MitC treatment) the amount of Svl eluted with choline did not increase (Fig.III.5B). This is in contrast to the continuous increase of choline extractable Svl of MitC-induced Δ*hol*Δ*lytA* (Fig.III.3C). Control PBS washes were unable to extract Svl and the absence of a Ts positive signal revealed no detectable cell lysis or cell contamination in any of the wash samples (Fig.III.5B). The ratio of the amount of choline-washed Svl at each time point by the corresponding amount of Ts detected in the cell pellet (which represents a measure of the number of cells washed; shown in Fig.III.5B, upper panel), renders the decreased levels of extracytoplasmic Svl in NaN_3-treated cells more perceptible, with 5.8-fold more Svl extracted in NaN_3-untreated cells at 80 min after MitC treatment (Fig.III.5C). Therefore, the decrease in cell wall Svl in the presence of NaN_3 could be attributed to a diminished export of phage lysin.

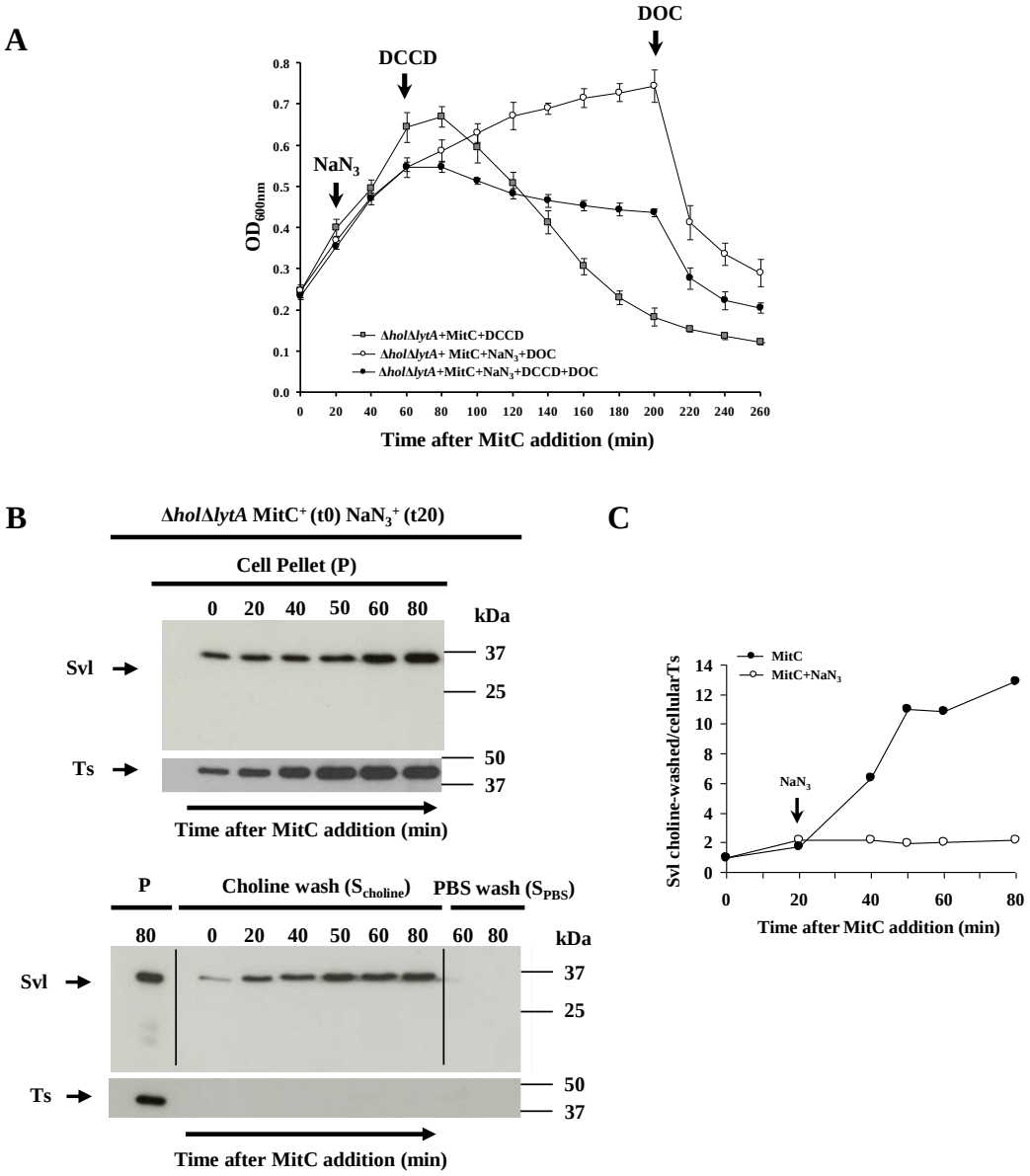


Figure III.5. Pneumococcal phage lysin export to the cell wall involves the Sec system. (**A**) Inhibition of Sec-mediated secretion prevents DCCD-induced lysis by phage lysin. SVMC28Δ*holΔlytA* cultures were treated with 0.1 µg/ml of MitC for phage excision at OD_{600nm}=0.2-0.25 (t=0 min). To a fraction of the cultures 5 mM of NaN_3 was added 20 min after induction, followed by 100 µM of DCCD at 60 min and 0.04% (w/v) of DOC at 200 min (indicated by arrows). In the other fraction, DCCD was omitted. As control for the effectiveness of DCCD-induced lysis, cultures were induced with MitC and treated only with DCCD after 60 min. Results are an average of a minimum of three independent experiments. Error bars represent 95% confidence intervals. (**B**) Phage lysin does not target to the cell wall when the Sec system is inhibited. SVMC28Δ*holΔlytA* was treated with MitC for phage induction (t=0 min) and with NaN_3 after 20 min. Equal aliquots were taken from cultures at 0, 20, 40, 50, 60 and 80 min. Cells were harvested by centrifugation and directly ressuspended in Tris 50 mM pH 7.5 (cell pellet fraction, P) or choline washed to remove choline binding proteins (choline wash fraction, $S_{choline}$). As control, cells collected at 60 and 80 min were also washed with PBS only (S_{PBS}). All fractions were tested by western blotting for the presence of Svl with anti-LytA antibody. P and S fractions were also tested with Ts (antibody 144,H-3) to control for the accumulation of cytoplasmic proteins throughout time and in the choline wash fraction to control for cytoplasmic contamination. In each lane, 10 µl of each P fraction and 45 µl of each S fraction were run. The mass of the molecular mass markers is indicated. Results are representative of a minimum of two independent experiments. (**C**) Comparison of phage lysin extracted by choline in the presence or absence of a functional Sec system. The quantity of phage lysin washed with choline from the cell wall of MitC-induced Δ*holΔlytA* cells treated with NaN_3 or left untreated was determined at each time point from the western blots shown in Fig.III.5B and 3C, respectively. The same was done for Ts present in the cell pellet under identical experimental conditions. The normalization of Svl-choline washed by the corresponding cellular Ts is graphically represented for each time point.

Surprisingly, despite Svl accumulation in the cell pellet of NaN_3-treated cells it was substantially less than in the absence of NaN_3 (Fig.III.5B and 3C, upper panels). In contrast, Ts increased in a similar fashion independently of NaN_3 treatment (Fig.III.5B and 3C, upper panels). In fact, a comparison between Svl and Ts in the cell pellet did not reveal an increase in Svl expression (data not shown), in contrast to the 6.7-fold increase discussed above (Fig.III.3C). The observed difference may reflect degradation of intracellular Svl unable to reach its final destination. Intracellular degradation of nonexported proteins was already described in previous studies with *Listeria monocytogenes*, in which MurA, transported by the accessory Sec system (SecA2), was not only absent from the cell wall in a *secA2* mutant but also in all subcellular fractions [57,58]. Collectively, these results point to the involvement of the host Sec system in the holin-independent export of pneumococcal phage lysin.

5. DISCUSSION

All *S. pneumoniae* phages rely on the holin-lysin system to achieve host lysis and release the new phage particles at the end of the lytic cycle [13-15,17,18]. It was proposed that, similarly to the majority of phages, pneumococcal phage lysins accumulate in the cytoplasm (hence designated endolysins) and are released by holin-mediated membrane disruption [1,2,13,15,16,29]. Here we provide evidence that the lysin of pneumococcal phage SV1 is targeted to the cell wall without requiring holins to permeabilize the membrane. Given the high structural and functional similarities between Svl and all other known pneumococcal phage lysins (exemplified by 85% to 97% nucleotide identity with lysins from phages EJ-1, MM1, HB-3 and VO1), our data point to the existence of exolysins in the majority of *S. pneumoniae* phages. Lysin export independent of holins has already been demonstrated for phages P1 and 21 of the Gram-negative *E. coli* and for phage fOg44 of the Gram-positive *Oenococcus oeni* [3,5,6]. However, in all of these cases a signal sequence for protein sorting is present, clearly indicating an extracytoplasmic pool of lysins [3,5,6]. The unusual feature of pneumococcal phage lysins is the absence of any known signal element for membrane translocation [13]. Using PSORT v3.0 (http://www.expasy.org) to predict protein subcellular localization, neither sequence motifs associated with a specific localization site, nor transmembrane regions or cleavable N-terminal signal peptides were detected in Svl, as reported for all other lysins of pneumococcal phages studied so far [13].

Phage lysins that target the cell wall of Gram-positive bacteria and that generally require holin-mediated access, frequently exhibit structural motifs for association with the cell surface (cell wall binding domains) that are responsible to direct the lytic enzymes to their substrate [59]. In *S. pneumoniae* phage lysins these also exist in the form of choline binding domains that recognize the choline residues [13,24] exclusively associated with the teichoic and lipoteichoic acids of the cell wall [13]. According to the exolysin model, where the pneumococcal phage lysin is continuously targeted throughout the lytic cycle, choline residues must also act as docking regions positioning and maintaining the enzyme already close to its substrate until the ideal lysis time. This is further strengthened by the presence of such domains in the bacterial cell wall hydrolase LytA [13,60], that is known to be localized and regulated in the pneumococcal cell envelope [19-23] (confirmed here by choline removal and treatment with pmf-dissipating agents). Building up an increasing amount of phage lysins at the surface, rather than targeting them to their site of action only at the end of the lytic cycle, may ensure a more rapid cell lysis once the lytic activity is triggered. It was further suggested that a thick peptidoglycan layer, characteristic of Gram-positive bacteria like *S. pneumoniae*, requires a

more extensive peptidoglycan degrading activity to promote lysis [3]. The accumulation of exolysins could thus represent an evolutionary advantage for pneumococcal phages.

Since the bulk of protein export across the cytoplasmic membrane is carried out in bacteria by the Sec pathway [56], we further investigated the involvement of this system on pneumococcal phage lysin targeting. Despite the absence of any known signal sequences, experiments with the Sec inhibitor NaN_3 suggest that Svl export occurs through the *S. pneumoniae* Sec pathway. In the presence of NaN_3, membrane pmf disruption induced by DCCD could no longer promote phage lysin activation since the accumulation of Svl at the cell surface was prevented. Additionally, exported Svl (eluted with choline from the cell wall) was indistinguishable by SDS-PAGE from Svl found in total cells, indicating that phage lysin present in the cell wall did not suffer proteolytic cleavage relative to its cytoplasmic form. Thus, phage lysin transport does not involve protein processing, which generally implies the absence of signal sequences [56], in agreement with the *in silico* prediction. The mechanism of this unusual Sec-dependent protein translocation still remains to be elucidated. Absence of secretory sequences concomitant with extracytoplasmic localization and Sec-dependent export was only reported in the mycobacteriophage Ms6 lysin LysA [55]. However, in this case the export is assisted by a phage chaperone-like protein (Gp1) that is encoded in the unusual lytic operon of Ms6. In contrast, SV1 and other pneumococcal phages lytic cassettes are less complex, not encoding other known functions besides holins and lysins [13-15,17,18]. Additionally, the analysis of SV1 genome as well as of other pneumococcal phages sequences available did not reveal Gp1-related proteins, strongly suggesting that the transport of pneumococcal phage lysin does not involve chaperone-like phage functions.

The results presented here may provide important clues for the still obscure transport mechanism of the bacterial autolysin. Similarly to Svl, bioinformatic predictions also indicate that LytA is deprived of motifs or signals that could position it outside the cytoplasm, although LytA was established early as a cell wall protein. Additionally, in common with the phage lysins, proteolytic cleavage of LytA was not detected ([20]; this work). We speculate that LytA may reach its final localization through the host Sec pathway that phages also seem to take advantage of to target their lysins. This hypothesis may be hard to address experimentally due to the ubiquitous expression of the autolysin (limiting experimental approaches blocking the Sec system) and the requirement of a functional Sec system for cell viability in many bacterial species [55,56]. Interestingly, this same mechanism may be also involved in the transport of few other peculiar choline binding proteins lacking specific targeting signals [61].

It was recently described in the Gram-positive bacterium *Listeria monocytogenes* the involvement of the accessory Sec system, dependent on SecA2 (a paralogous of SecA of the

canonical Sec pathway), in the export of several proteins without any obvious signals for bacterial surface targeting [58]. Nonetheless, the features of this subset of SecA2-exported proteins that target them for export by the SecA2 pathway remain unknown. It is remarkable that one of those peculiar *L. monocytogenes* proteins is the *N*-acetylmuramidase MurA that is, like the autolysin LytA and the pneumococcal phage lysins, a cell wall hydrolase [56]. Moreover, in this species, another murein hydrolase p60 is also exported through SecA2 but in this case it possesses a signal sequence [57,58]. Interestingly, SecA2 is also functional in *Streptococcus* species and a *secA2* gene was detected in *S. pneumoniae* in a very high proportion of strains [37,38,56,62]. Thus, pneumococcal phage lysins, as well as LytA, could be secreted in a SecA2-dependent manner. It should be pointed out that very little is known about this type of transport in pneumococcus and in other streptococci the proteins reported to be exported via SecA2 are characterized by the presence of signal sequences [56,63]. Since SecA2 is an ATPase that provides energy for translocation like SecA, NaN_3 should also result in its inhibition [38,56], rendering impossible the distinction between the two Sec pathways by this methodology. Further experiments are therefore needed to determine which Sec system is specifically involved.

In the context of phage exolysins, the previously demonstrated permeabilizing action of holins on the *S. pneumoniae* membrane [14,15,18,29] is not necessary to allow the passage of phage lysins from the cytoplasm onto the cell wall, as suggested before [13,15,16,29]. In fact, we showed that it is crucial to trigger the collapse of the membrane pmf that leads to lysins activation. We observed that a significant phage lysin secretion occurs before lysis is achieved, indicating that Sec-mediated export of the lysin is not sufficient for lysis. But when pmf is depleted before the normal lysis time (for instance by the addition of DCCD), complete lysis occurred through Svl activation. For the other described phages encoding exolysins, it was also argued that membrane pmf disruption achieved by the holins activate the exported lysins [5,6,9,55]. Still, regardless of the phage lysin cellular localization (cytoplasmic or extracytoplasmic) that determines the way the holins trigger their action (permeabilization or pmf collapse), in all holin-lysin systems it is the holins that signal the lysins when to play their part in the release of phage progeny.

In phage 21, the secreted SAR lysin is associated with a pinholin that, as opposed to the large-hole-forming λ-like holins, forms channels not sufficiently large for lysins to pass through [1,6,64,65]. In fact, this pinholin is unable to complement the lysins of phage λ in promoting lysis [6]. Although we did not specifically evaluate the size of SV1 holin lesions, holins of other *S. pneumoniae* phages were shown to form holes large enough to allow lysin passage [15,29]. Additionally, inspection of the amino acid sequence of both SV1 holins did not reveal an N-

terminal (transmembrane) SAR domain (rich in residues that are weakly hydrophobic), a feature described for the pinholin of phage 21 [8,65]. However, exolysins not associated with a pinholin have been described [6]. The holin of phage P1, which is paired with the SAR lysin Lyz, is in fact a canonical holin [6]. Curiously, the Gp4 holin of the exolysin-carrying mycobacteriophage Ms6 has in its primary sequence characteristics of pinholins, although it is able to promote the release of the cytoplasmic λ lysin but not of the Ms6 lysin [66].

Continuous localization of the phage lysin to the cell wall requires that it be held in an inactive state, which we showed to be related to the energized membrane. However, it remains unknown how membrane pmf is converted into a suppressive modulator of Svl or how the inactive state is subverted by the dissipation of the cytoplasmic membrane electrochemical gradient. Since we observed that the bacterial LytA is also sensitive to the energy status of the membrane and given the dependence of LytA and phage lysins on choline binding for catalytic activity [13,60,67], pmf may influence the activities of both the phage lysin and bacterial autolysin in a similar way. It is accepted that the interaction with the membrane-bound lipoteichoic acids regulates LytA activity [19,23] and it was even observed that LTA inhibited the activity of the Cpl-1 lysin of phage Cp-1 [68]. Furthermore, it was suggested that the membrane establishes a specific conformation of LTAs in Gram-positive bacteria since, for instance, variations of the ionic strength of the medium result in LTA conformational change [69]. Thus, loss of the membrane pmf promoted by the holins may induce structural and chemical rearrangements in the cell envelope that may result in the abolishment of the inhibitory activity of LTA over both lysins. The holin lesions could also release some activator factor from the membrane. However, preliminary results do not seem to support this hypothesis since no lysis was observed in Δ*hol*Δ*svl* or Δ*hol*Δ*lytA* strains treated with MitC for 80 min (allowing LytA or Svl expression) with the supernatant of Δ*svl*Δ*lytA* cells, carrying functional holins, treated with MitC for 140 min (at which time, lysis is completed in the wild-type culture). The possibility that resulting pH changes may specifically influence lysin control should also be considered. Indeed, in *Bacillus subtilis* it was shown that the cell wall has a low local pH sustained by the membrane pmf and its abolishment results in cellular lysis [70]. Further experiments are required to clarify these issues.

In conclusion, our data supports the inclusion of the majority of pneumococcal phages in the class of phages carrying exolysins, greatly increasing the number of phages with this lysis mechanism and indicating that these may be much more frequent than previously thought. In these phages, membrane pmf dissipation by the holins is necessary for lysin activation. In *S. pneumoniae* this is also sufficient to trigger the externalized major autolysin LytA that was previously shown to contribute significantly to phage progeny release.

6. ACKNOWLEDGEMENTS

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7. SUPPLEMENTARY DATA

The following supplementary data are available for this chapter:

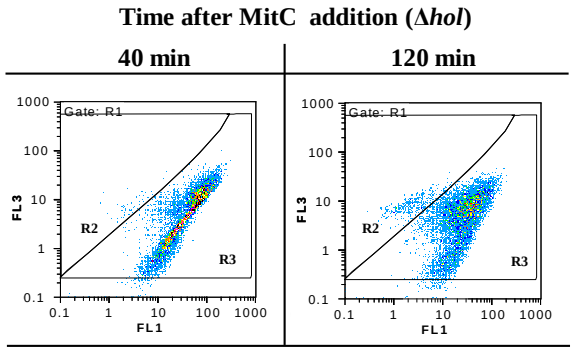

Figure III.S1. Effect of holin deletion on SVMC28Δ*hol* membrane integrity. SVMC28Δ*hol* strain (lacking holins but with functional LytA and Svl) was grown until OD_{600nm}=0.2-0.25 and 0.1 μg/ml of MitC was added to induce phage excision (t=0 min). Cells were collected after 40 and 120 min and tested for membrane permeabilization by flow cytometry following staining with a mixture of Syto 9 and propidium iodide (PI). Gate R2 corresponds to the membrane damaged population, while gate R3 corresponds to the population with intact membrane. Both gates were designed over gate R1, which includes the total stained population. At 120 min, the large majority of cells maintains membrane integrity. Results are representative of a minimum of two independent experiments.

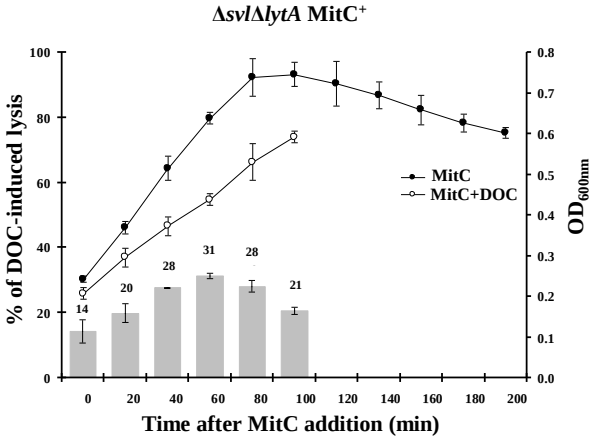

Figure III.S2. Kinetics of DOC-induced lysis after phage induction in SVMC28Δ*svl*Δ*lytA* strain. SVMC28Δ*svl*Δ*lytA* was grown until OD_{600nm}=0.2-0.25 and 0.1 μg/ml of MitC was added to induce phage excision (t=0 min). Cultures were treated with DOC [final concentration of 0.04% (w/v)] at 20-min intervals after phage induction. OD_{600nm} is indicated before (full symbols) and 20 min after DOC addition (open symbols) and DOC-triggered lysis is expressed as percentage of optical density drop (values are shown above the bars). The residual lysis observed in SVMC28Δ*svl*Δ*lytA* $MitC^+$ may be attributed to other bacterial lysins. A rough comparison with SVMC28Δ*hol*Δ*lytA* $MitC^-$ (Fig.III.2A2) seems to indicate that in this case the contribution of the phage lysin accumulated due to spontaneous phage induction to the residual lysis is only apparent from 80 min onwards. Analysis of a MitC-untreated SVMC28Δ*hol*Δ*svl*Δ*lytA* mutant is needed to better evaluate this effect. Results are an average of a minimum of three independent experiments. Error bars represent 95% confidence intervals.

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CHAPTER IV

PROPHAGE SPONTANEOUS ACTIVATION PROMOTES DNA RELEASE ENHANCING BIOFILM FORMATION IN *STREPTOCOCCUS PNEUMONIAE*

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1. SUMMARY

Streptococcus pneumoniae (pneumococcus) is able to form biofilms *in vivo* and previous studies propose that pneumococcal biofilms play a relevant role both in colonization and infection. Additionally, pneumococci recovered from human infections are characterized by a high prevalence of lysogenic bacteriophages (phages) residing quiescently in their host chromosome. We investigated a possible link between lysogeny and biofilm formation. Considering that extracellular DNA (eDNA) is a key factor in the biofilm matrix, we reasoned that prophage spontaneous activation with the consequent bacterial host lysis could provide a source of eDNA, enhancing pneumococcal biofilm development. Monitoring biofilm growth of lysogenic and nonlysogenic pneumococcal strains indicated that phage-infected bacteria are more proficient at forming biofilms that is their biofilms are characterized by a higher biomass and cell viability. The presence of phage particles throughout the lysogenic strains biofilm development implicated prophage spontaneous induction in this effect. Analysis of lysogens deficient for phage lysin and the bacterial major autolysin revealed that the absence of either lytic activity impaired biofilm development and the addition of DNA restored the ability of mutant strains to form robust biofilms. These findings establish that limited phage-mediated host lysis of a fraction of the bacterial population, due to spontaneous phage induction, constitutes an important source of eDNA for the *S. pneumoniae* biofilm matrix and that this localized release of eDNA favors biofilm formation by the remaining bacterial population.

2. INTRODUCTION

Biofilms, the most frequently encountered physiological form adopted by microorganisms, are surface-adapted communities that constitute a protected mode of bacterial growth allowing survival in hostile environments [1-4]. Recent studies demonstrated the potential of *Streptococcus pneumoniae* to produce biofilms *in vivo* [5,6]. Pneumococcal biofilms were indeed detected on affected tissues in patients with chronic rhinosinusitis [6], children with otitis media [5], as well as a chinchilla model of otitis [7]. The presence of these communities at the site of infection implicates them in these disorders, although their significance in the infection process is a matter of debate. Two recent studies have failed to find an association between the ability to form biofilms and whether the isolates had been recovered from asymptomatic carriers or caused invasive infections [8,9]. Moreover, a mouse model of invasive infection failed to show any association between the capacity to cause bacteremia and the ability of the strains to form robust biofilms [8]. Although these two studies question the role of biofilms in determining the invasive potential of pneumococci, the transcriptional profile of several known virulence-related genes in *S. pneumoniae* isolated from lungs and brains of infected mice is similar to that in biofilms formed *in vitro*, suggesting a possible biofilm-like state of *S. pneumoniae* associated with tissues [10]. In addition, a link was established between pneumococcal biofilm formation and the asymptomatic colonization of the nasopharynx [11], the most frequent state of pneumococci. Overall, these studies highlight the importance of studying *S. pneumoniae* biofilms, particularly of identifying the factors that influence the formation of these structures.

Bacterial biofilms are encased within an extracellular matrix consisting of polysaccharides, proteins and nucleic acids [1]. Although polysaccharides and proteins are important components, the role of extracellular DNA (eDNA) as a critical element of the matrix is increasingly recognized, both in providing structural stability as well as protection against antimicrobial agents [12-15]. In Gram-positive bacteria, such as *Enterococcus faecalis* and *Staphylococcus epidermidis*, autolysins (bacterial murein hydrolases) were recently implicated in biofilm formation, apparently by mediating bacterial lysis with the consequent release of eDNA [12,16-18]. Pneumococcal cells are characterized by the presence of a major autolysin LytA, an *N*-acetylmuramoyl-L-alanine amidase, which is responsible for the unusual property of massive cellular lysis displayed in the stationary phase of liquid cultures [19]. Furthermore, cell lysis dependent on LytA was also detected upon competence development, which results in DNA release into the medium [20,21]. The observation that *S. pneumoniae* biofilm formation is influenced by the presence of eDNA [22,23] and that LytA mutants have a decreased capacity

to form biofilms [23], hints that LytA-induced pneumococcal lysis could be related to biofilm formation through the release of eDNA.

In addition to autolytic events, cell lysis in *S. pneumoniae* can also be mediated by lysogenic phages, which have a high prevalence (76%) in isolates associated with infection [24,25]. During lysogeny, the prophage is integrated in the bacterial chromosome being replicated as part of the host genome. Upon induction, the repressed lysogenic state shifts to lytic growth with the production of viral particles and subsequent phage-mediated host lysis to release the phage progeny [24,26]. It was recognized early that free phages can be found in cultures of lysogenic bacteria in the absence of a known inducing agent, indicating that some prophages spontaneously enter the lytic cycle [27]. Spontaneous phage induction seems to be a common feature of lysogeny, being nonspecific of the phage or the bacterial host, although the factors that promote spontaneous induction, either *in vitro* or *in vivo*, are poorly understood. Recent studies showed that this natural phenomenon may contribute to pathogenicity in *Salmonella* [28], increasing the awareness of the potential importance of lysogeny in the context of infection. This spontaneous phage release occurs obviously at low levels, and the phage titer observed is orders of magnitude less than the one produced when the same bacteria are treated with an inducing agent [26,29,30].

Whether agent-induced or spontaneous, it was believed that phages of *S. pneumoniae* relied exclusively on their own lysins to hydrolyze host cell wall peptidoglycan and release the phage progeny [31]. Recently, it was shown that pneumococcal lysogenic phages achieve an optimal exit strategy by orchestrating the coordinated action of the phage-encoded lysin and the bacterial major autolysin LytA [32].

Inevitably, prophage activation results, through bacterial lysis, in the release of the cellular components to the extracellular medium. Since eDNA is increasingly recognized as a critical element for biofilm formation, we hypothesized that spontaneous induction of lysogenic phages could have a positive effect on pneumococcal biofilms. To test this, we have evaluated biofilm formation and eDNA release of isogenic strains differing in carriage of a prophage and having functional or being deleted in the major phage and bacterial lysins.

3. MATERIAL AND METHODS

Bacterial strains, culture conditions and DNA manipulations

Bacterial strains SVMC28 and R36A were obtained from the Rockefeller University collection (A. Tomasz). R36A is a nonlysogenic, nonencapsulated strain [24]. SVMC28 is an encapsulated (serotype 23F) clinical isolate, lysogenic for phage SV1 encoding the Svl phage lysin [32]. R36AΔ*lytA* was kindly provided by S. Filipe. SVMC28 derived mutants SVMC28Δ*svl*, SVMC28Δ*lytA* and SVMC28Δ*svl*Δ*lytA* belong to the Faculdade de Medicina de Lisboa collection. The SV1-lysogenized strains R36AP, R36APΔ*lytA*, R36APΔ*svl* and R36APΔ*lytA*Δ*svl* are also from the Faculdade de Medicina de Lisboa collection. All strains were described previously [32]. All *S. pneumoniae* strains were grown in a casein-based semisynthetic medium (C+Y) at 37ºC without aeration or in tryptic soy agar (TSA) (Oxoid, Hampshire, England) supplemented with 5% (v/v) sterile sheep blood incubated at 37ºC in 5% CO_2. For overnight cultures, pneumococcal mutant strains were grown in the presence of 2 μg/ml erythromycin or 4 μg/ml chloramphenicol (Sigma, Steinheim, Germany) or both, as appropriate. After selective growth, the culture was diluted 1:100 in fresh medium and grown until the appropriate optical density. Chromosomal DNA from *S. pneumoniae* strain R36A was isolated following previously described procedures [33]. Sperm salmon DNA was purchased from Invitrogen Co. (Carlsbad, California, USA).

Biofilm biomass quantification

Biofilm formation was determined by the ability of cells to grow adherent to 96-well flat-bottom polystyrene microtiter plates (Nunc™, Roskilde, Denmark) in static conditions. Cells were grown in C+Y medium, with selective antibiotic when necessary, to an optical density measured at 600 nm (OD_{600nm}) between 0.5 and 0.6 and then diluted 1:4 in fresh medium to a final volume of 200 μl per well. Microtiter plates were incubated at 37 ºC and biofilm mass was determined by staining with crystal violet [23] and measuring the OD_{595nm} using a plate reader (Tecan Infinite M200 with i-control™ software V1.40). The incubation times at which the biomass was quantified were selected based on preliminary experiments in order to monitor the dynamics of biofilm growth and dispersal. Shorter time intervals were selected when biofilm mass showed steeper variations. The incubation times at which biomass was quantified were 6 h, 12 h, 18 h, 20 h, 24 h, 26 h, 28 h and 30 h of incubation. A control with only C+Y medium was also done for all time points and the values were subtracted to those measured for all strains.

Quantitative determination of biofilm formation was also evaluated in the presence of deoxyribonuclease I (DNase I) and DNA, incorporated in the medium. DNase I (Sigma, Steinheim, Germany) was used at a final concentration of 0.5, 5 or 50 μg/ml and biofilm mass was measured after 24 h of incubation. DNA from R36A strain or salmon sperm was added at 10, 100 or 1000 ng/ml to the medium and determination of biofilm formation was carried out 24 h post incubation. Values obtained from medium supplemented with DNAse I and DNA were subtracted in all strains. To test if DNA was important in biofilm adherence, the plate wells were incubated with 1000 ng/ml of R36A DNA overnight at 4ºC to condition the plastic surface. The solution was then discarded and the biofilm was seeded as described before. Biofilm mass was determined at 24 h post incubation. To determine if the impact of DNA on biofilm formation was due to a structural role, DNA from R36A was broken by sonication for 5 min at 0.63 A and 50–60 Hz in a Transsonic T570 (Elma, Germany), and added to the medium at 1000 ng/ml. DNA fragmentation was confirmed by agarose gel electrophoresis. Biofilm formation was compared to biofilms grown in the presence of 1000 ng/ml of intact DNA.

Biofilm colony forming units (CFU) assays

Biofilms were grown in 96-well plates at 37ºC as described for the biofilm biomass quantification assay. CFUs were determined at the selected time points between 6 h and 30 h of incubation. Liquid medium with bacteria was gently removed from the wells, which were washed twice with phosphate buffered saline (PBS) 1x, pH 7.2 (Invitrogen, Grand Island, New York) to eliminate unbound bacteria without disturbing the adherent biofilm. 200 μl of PBS were then added to each well and biofilms were scraped thoroughly, including well edges. The well contents were recovered and the total CFU number was determined by serial dilution and plating on appropriate media.

To test the effect of DNase I and DNA on biofilm development, DNase I was added to the growth medium to a final concentration of 0.5, 5 or 50 μg/ml. After 24 h of incubation at 37ºC, CFUs were determined as described above. When using DNA to evaluate its effect on biofilm formation, DNA from salmon sperm was added to the growth medium at a final concentration of 1000 ng/ml and CFUs were determined as described above.

Phage plaque assays

Plaque assays were performed as described elsewhere [32]. In detail, basal plates were made by pouring C+Y medium with 170 U catalase per ml and 1% agar into Petri dishes. A lawn

culture of R36A strain grown to an OD_{600nm} of 0.2 was mixed with soft agar containing C+Y supplemented with 170 U catalase per ml and 0.35% agar. The entire mixture was spread onto basal plates. After hardening, phage preparations were applied in 10 µL aliquots directly on the soft agar with the R36A indicator strain. Incubation was performed at 30 ºC for 18 h. To obtain the phage preparation, at the chosen time points after biofilm seeding each well was scraped thoroughly including well edges. The harvested biofilms were filtered through a 0.45 µm-pore-size membrane followed by filtering with a 100 000 MWCO polyethersulfone membrane (Vivaspin concentrator, Sartorius Stedim biotech, Goettingen, Germany), that retains and concentrates the SV1 phage [32]. The phage concentrate was stored at 4ºC for a maximum of 24 h until usage. The filtrate containing proteins < 100 KDa, that could cause bacterial lysis such as LytA and bacteriocins, was also used to eliminate the possibility that lysis of the indicator strain was caused by bacterial products and not caused by phage infection. Images of the plates were acquired with the high-performance stereo-microscope Leica MZ7.5 (Leica Microsystems, Germany) and the number of plaque forming units (PFUs) was counted manually by visual inspection of the image.

Confocal laser scanning microscopy (CLSM)

Biofilms were stained by using a Live/Dead BacLight bacterial viability kit (Invitrogen, Carlbad, USA) and examined by CLSM. Syto 9/PI labelled biofilms allowed for monitoring the viability of bacterial populations as a function of the membrane integrity of the cell. Cells with a compromised membrane (dead cells) will stain red whereas cells with an intact membrane (live cells) will stain green. Whenever DNA and DNase I effects were tested, the medium was supplemented before biofilm seeding (t=0). In all experiments, biofilms were analyzed after 24 h of incubation. Images were acquired on a Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany) using a PlanApochromat 63x/1.4 objective for cell viability assays and a C-AproChromat 40x/1.2. Syto 9 fluorescence was detected using the 488 nm laser line of an Ar laser (45 mW nominal output) and a BP 505–550 filter. PI fluorescence was detected using a DPSS 561 nm laser (15 mW nominal output) and a LP 575 filter. For imaging, the laser power was attenuated to 1-2% of its maximum value. The pinhole aperture was set to 1 Airy unit.

Purification and quantification of eDNA

Biofilms were grown in 96-well plates at 37ºC as reported above. eDNA was purified from 24-h biofilms exactly as previously described [34]. eDNA was quantified by real-time PCR using

the primes gdh-up (5´-ATGGACAAACCAGCNAGYTT) and gdh-dn (5´-GCTTGAGGTCCCATRCTNCC) and spi-up (5´-TTATTCCTCCTGATTCTGTC) and spi-dn (5´-GTGATTGGCCAGAAGCGGAA), amplifying the *gdh* and *spi* genes used for multilocus sequence typing (MLST), respectively. These are housekeeping genes located far apart in the R36A chromosome. PCRs were performed on nondiluted samples with the SYBR Green Jump Start Taq Ready Mix (Sigma, Steinheim, Germany), according to the manufacturer's recommendations. Purified R36A genomic DNA at known concentrations was also subjected to quantitative real-time PCR with each primer pair to generate a standard curve used to calculate the concentration of eDNA in the unknown samples. PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, California, USA). To account for potential differences in biomass, the average OD_{595nm} of each biofilm was determined and used to calculate the relative OD_{595nm} of each biofilm with respect to the OD_{595nm} of the wild-type R36A biofilm. The nanogram of eDNA per relative biomass of each biofilm was then calculated by dividing its total eDNA (ng) by its relative OD_{595nm}.

4. RESULTS

Lysogenic phages enhance biofilm development

In order to evaluate the impact of lysogeny in biofilm formation a well-established *in vitro* system, based on an abiotic surface as the growth substrate, was used allowing proper investigation of the initial stages of biofilm formation [8-10,22,23]. We started by monitoring biofilm development of the isogenic pair of *S. pneumoniae* strains R36A and R36AP, which differ only in the presence of a prophage (R36AP is a lysogen of phage SV1). Biofilm growth was followed at specific time points between 6 h and 30 h of incubation by biomass quantification and viable cell counts (Fig.IV.1A and B). The evaluation of cell viability by CFUs was consistent with biomass quantification obtained by crystal-violet staining. The biofilm of the lysogenic strain R36AP reaches its maximal development at 24 h and from that time onwards a decrease in biomass occurs. We reasoned that this decrease is inherent to the experimental conditions used, probably due to nutrient depletion, accumulation of toxic substances or intrinsic properties of the biofilm. In contrast, for the wild-type nonlysogenic strain R36A the highest biofilm mass values are registered at 26 h, decreasing afterwards in a behavior similar to that of strain R36AP. This observation is consistent with a slower biofilm growth of strain R36A, resulting in delayed development. The lysogenic strain showed improved biofilm growth at all time points and also a higher maximal biofilm mass than its nonlysogenic parent. In agreement with these findings, images of CLSM show denser and thicker biofilms for R36AP (Fig.IV.2A and B). Since the lysogen R36AP is indistinguishable from its parental strain R36A in planktonic growth [32], the differences observed must be attributed to the influence of the lysogenic phage on biofilm formation.

Spontaneous prophage induction enhances biofilm development due to host lysis

It was previously shown that the main pneumococcal autolysin LytA is important in normal biofilm development since its inactivation resulted in diminished biofilm formation, possibly by a mechanism dependent on its regulated lytic activity [23]. Thus, autolytic events may be helpful in the establishment of robust *S. pneumoniae* biofilms. It is well known that spontaneous phage induction results in the lysis of a fraction of the bacterial population [29] and we speculated that such induction could also occur within pneumococcal biofilms. Accordingly, the enhanced biofilm formation of the lysogenic strain R36AP could be explained by limited phage-triggered lysis. To test this hypothesis, we compared biofilm development of the lysogenic strain R36AP to that of the derived mutants for phage lysin Svl (strain

R36APΔ*svl*), bacterial autolysin LytA (R36APΔ*lytA*) or both lysins (R36APΔ*lytA*Δ*svl*). As shown in Fig.IV.1A and B, biofilm growth is significantly impaired in the absence of the phage lysin with a shift in the biofilm biomass peak from 24 h to 26 h, analogous to the growth pattern observed for the nonlysogenic strain (R36A). A similar behavior was observed for the lysogen in the absence of the bacterial autolysin. In fact, the presence of at least one lysin is essential, as the double mutant was largely deficient in biofilm formation. Accordingly, the nonlysogenic R36AΔ*lytA* strain is also severely impaired in biofilm formation, supporting an important role of bacterial lysis in biofilm formation. This inability to form biofilms is not due to a growth defect since all mutants presented identical planktonic growth to the parent lysogen [32]. These results are consistent with the hypothesis that the positive impact of prophages in pneumococcal biofilm development is due to spontaneous induction of the lytic cycle resulting in cell lysis.

In order to confirm if phage induction was indeed occurring in the biofilm, we measured the phage particles released during biofilm development of strain R36AP by determining the number of PFUs throughout biofilm growth (Fig.IV.1C). We observed the presence of phages in the biofilm at all time points, indicating that spontaneous phage induction is occurring continuously and paralleling the increase in viable cells. A substantial increase in the number of PFUs coincides with the peak of biofilm development (Fig.IV.1A and B), indicating increased phage induction at the later stages of biofilm formation. This higher phage induction is not due to a massive triggering of the phage lytic cycle related to this stage of biofilm growth since no increase of PFUs concomitant with biofilm dispersal was observed. Altogether, these results strongly support a role of spontaneous phage induction during biofilm development in the different phenotype shown by lysogenic strains.

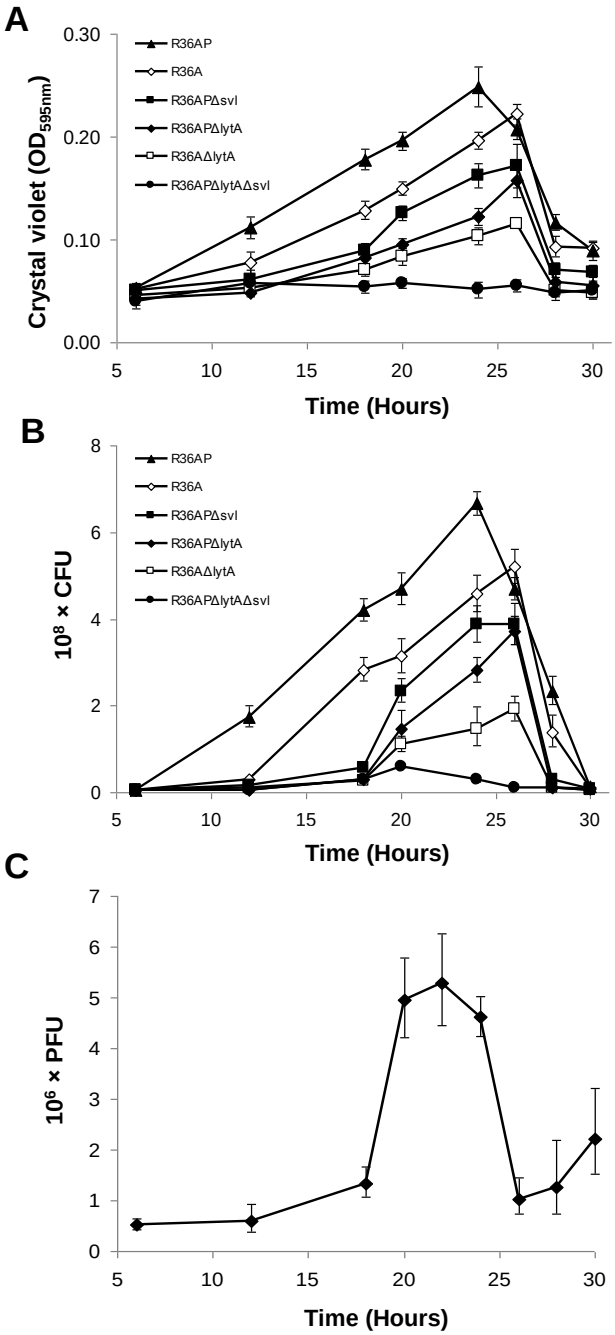

Figure IV.1. Effect of lysogeny and phage induction in *Streptococcus pneumoniae* biofilm development. (**A**) Biofilm development monitored as biomass from 6 h to 30 h. R36A nonlysogenic strain; R36AP lysogenic derivative of R36A; R36APΔ*svl*, R36APΔ*lytA*, R36AΔ*lytA* and R36APΔ*lytA*Δ*svl* are mutants in which the phage lysin (Svl), the bacterial autolysin (LytA) or both were deleted. Results are an average of 9 independent replicates. (**B**) Biofilm development monitored as CFUs from 6 h to 30 h. The strains are the same indicated in panel A. Results are an average of 6 independent replicates. (**C**) The presence of phage in the R36AP biofilm was determined by the production of plaques on R36A. PFUs were determined throughout biofilm development from 6 h to 30 h. Results are an average of 2 to 7 independent replicates for each time point. In all panels error bars represent 95% confidence intervals for the sample mean.

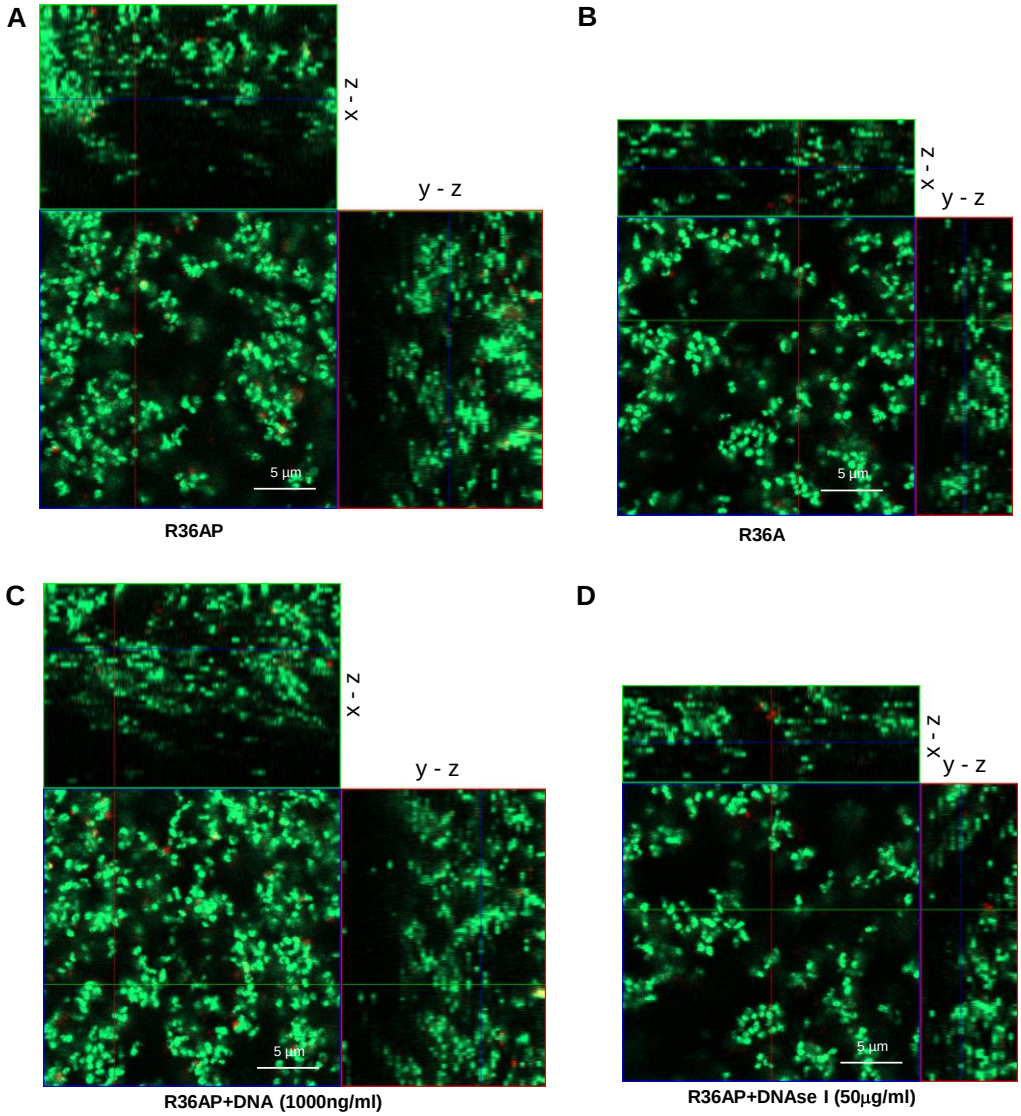

Figure IV.2. Confocal laser scanning microscopy images of R3A6P and R36A biofilms. Staining was done with Syto 9/PI (Live/Dead BacLight Bacterial Viability kit) and images were acquired at 630x amplification. Live cells internalize only Syto 9 (fluorescing green) whereas dead cells allow the uptake of PI (fluorescing red). The large images are optical sections of top views and the small images to the right and above are optical sections of side views. The depth of the biofilm is indicated by the height of the *z*-stack. The inset scale bar represents 5 µm. (**A**) Biofilm formed by the lysogenic strain R36AP. (**B**) Biofilm of the nonlysogenic strain R36A. (**C**) The biofilm was grown in the presence of salmon sperm DNA at 1000 ng/ml. (**D**) The biofilm was grown in medium supplemented with DNase I at 50 µg/ml. In all panels the results are representative images of 3 independent experiments and biofilm growth was evaluated at 24 h.

Released eDNA through phage-mediated lysis is a key factor for biofilm enhancement

Extracellular DNA is an essential matrix component produced by many bacterial species during biofilm development [12,15,16,35,36]. Therefore, we hypothesized that in *S. pneumoniae* phage-mediated lysis of a fraction of the bacterial population within the biofilm could provide an extra source of eDNA for incorporation in the biofilm matrix.

We performed a DNase I susceptibility assay by incubating this enzyme for 24 h with R36A and R36AP in conditions allowing biofilm formation. A gradient of DNase I concentrations, ranging from 0.5 to 50 µg/ml was used. Biofilm biomass quantification indicates that DNase I reduces biofilm formation in a dose-dependent way (Fig.IV.3A). As expected, the biomass reduction is directly related to a decrease of viable cells in the biofilm (data not shown). The effect of DNase I is similar in R36A and R36AP biofilms, however, R36AP biofilms always show a higher biomass than R36A biofilms at all tested DNase I concentrations, suggesting that R36AP biofilms are richer in eDNA. In fact, the biomass of the R36AP biofilm incubated with 0.5 µg/ml of DNase I, is similar to that of the R36A biofilm incubated in the absence of DNase I, indicating that the presence of this enzyme reduced the extra eDNA present in the R36AP matrix, resulting in a biofilm similar to that of R36A. Taken together, the data enable an argument to be made for the beneficial effect of lysogenic phages in biofilm development due to an increased presence of eDNA in the matrix.

To further explore this potential role of eDNA on biofilm development, we decided to determine the effect of the addition of external DNA to the medium since the time of seeding, on biofilm mass measured at 24 h of growth. DNA was extracted from the R36A strain (homologous DNA) and used at a final concentration of 10, 100 and 1000 ng/ml. To rule out any specific effect of pneumococcal DNA, the same experiments were repeated using DNA isolated from salmon sperm (heterologous DNA). As shown in Fig.IV.3B, incubation with DNA since biofilm seeding enhances biofilm development in a dose-dependent manner, with a significant effect detected with as little as 10 ng/ml. This biomass increase parallels the number of viable cells in the biofilm (data not shown). Moreover, this effect is observed with both homologous and heterologous DNA, indicating that this was due to an intrinsic property of the DNA molecule and independent of the exact nucleotide sequence and donor organism.

Microscopy was used to explore the differences between untreated R36AP biofilms and those treated with 50 µg/ml of DNase I and 1000 ng/ml of DNA. In agreement with the results obtained by biomass quantification, treatment with DNase I resulted in sparser and thinner biofilms when compared to control (Fig.IV.2A and D). On the other hand, supplementation of the medium with DNA resulted in a more densely packed and thicker biofilm (Fig.IV.2A and C). These results further support that the limited lysis promoted by lysogenic phages during biofilm development leads to higher eDNA release resulting in stronger biofilm growth.

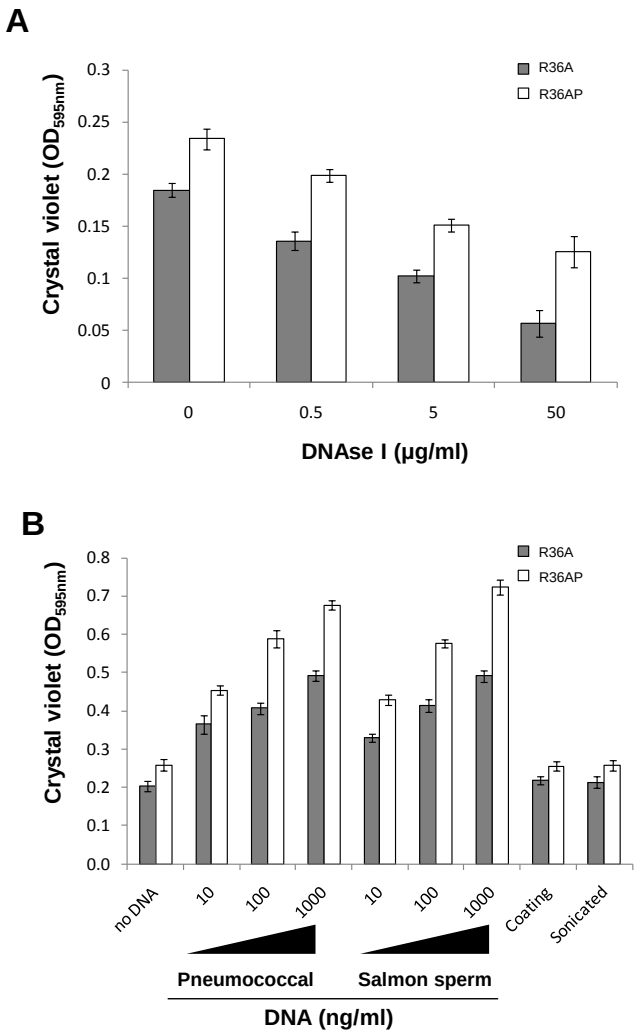

Figure IV.3. Effect of DNAse I and DNA on biofilm mass. (**A**) The lysogenic strain R36AP and its nonlysogenic parent R36A were exposed from seeding to DNAse I at final concentrations of 0.5, 5 and 50 µg/ml. Biofilm mass was quantified after 24 h of incubation. (**B**) R36AP and R36A were exposed from seeding to DNA from R36A or salmon sperm at final concentrations of 10, 100 and 1000 ng/ml. In separate experiments, the effect on biofilm development of coating the wells with 1000 ng/ml of R36A DNA prior to seeding and the addition of sonicated R36A DNA at 1000 ng/ml since the time of seeding was also determined. Biofilm mass was assessed at 24 h of incubation. In all panels, the results are an average of 9 independent replicates and error bars represent 95% confidence intervals for the sample mean.

Due to the different kinetics of biofilm development of the lysogenic and nonlysogenic strains, we wanted to clarify if the role of DNA was only critical in the initial steps of biofilm establishment (initial cell attachment) or if its presence was necessary throughout the subsequent early phases of biofilm development. To this end, the wells where the biofilms were grown were precoated with DNA followed by incubation of the bacteria in DNA-free medium. After 24 h, biofilm mass was similar to the uncoated control (Fig.IV.3B), indicating that the observed DNA effect is not related with the initial adherence process.

Furthermore, we also examined whether DNA acts as a structural component of the biofilm or if the availability of extra nutrients, due to the presence of DNA in the medium, could explain the enhanced biofilm development. With that in mind, we grew biofilms in the presence of sonicated DNA and compared them with biofilms formed in the presence of intact DNA, using both homologous and heterologous DNA. In the presence of fragmented DNA, biofilm development assessed at 24 h was similar to that of biofilms grown in the absence of DNA and substantially less to that observed with intact DNA (Fig.IV.3B). This data revealed that large DNA fragments were essential for the enhancement of biofilm growth and suggested that DNA had an important structural role in biofilm architecture.

To confirm if a higher eDNA release due to phage spontaneous induction is related to a strong biofilm development we determined the actual eDNA released into the biofilm of each strain, after 24 h of growth, by quantitative real-time PCR. Strains with a higher capacity to form biofilm (R36AP, R36A, R36APΔ*svl* and R36APΔ*lytA*) contain significantly more eDNA in comparison to the almost undetectable levels present in R36APΔ*lytA*Δ*svl* and R36AΔ*lytA*, two strains with poor biofilm forming capacity (Fig.IV.4A). The marked difference observed between lytic and nonlytic strains suggest that lytic events resulting in eDNA release have a strong positive impact in biofilm development.

DNA release upon phage induction is dependent on lysis. So, we reasoned that the addition of external DNA to biofilms of the mutant strains R36APΔ*svl*, R36APΔ*lytA* and R36APΔ*lytA*Δ*svl* would allow the development of more robust biofilms. Indeed, when the mutant strains were given exogenous DNA, biofilm development was strongly increased in R36APΔ*svl* and R36APΔ*lytA* (Fig.IV.4B and S1A). The addition of a large excess of DNA overcomes the impairments created by the ablation of either the phage or bacterial lysins, with the formation of more biofilm in the presence of DNA by these mutants than that observed when R36AP, where both lysins are functional, was incubated in the absence of exogenous DNA (Fig.IV.4B and S1A). As pointed out previously, the mutant lacking both lysin activities (R36APΔ*lytA*Δ*svl*) was incompetent to form stable biofilms and, even in the presence of excess DNA, failed to recover to the R36AP level. Thus the addition of DNA does not fully overcome the abolishment of the two major lysins present in R36AP. This is in contrast to R36AΔ*lytA* that responds well to the addition of external DNA. Although both mutants present similar amounts of eDNA (Fig.IV.4A), the R36AΔ*lytA* strain forms more biofilm biomass than strain R36APΔ*lytA*Δ*svl* (Fig.IV.1A) and this effect is even more pronounced in the number of viable bacteria in the biofilm (Fig.IV.1B). It has been previously shown that even when phage and bacterial lysins are deleted, phage induction decreases cell viability as phages express holins that collapse the cell membrane potential resulting in host cell death [32]. Thus, this

difference in cell viability between R36AΔ*lytA* and R36APΔ*lytA*Δ*svl* may be sufficient to compromise the enhancement of biofilm development in the presence of added DNA observed in the later strain.

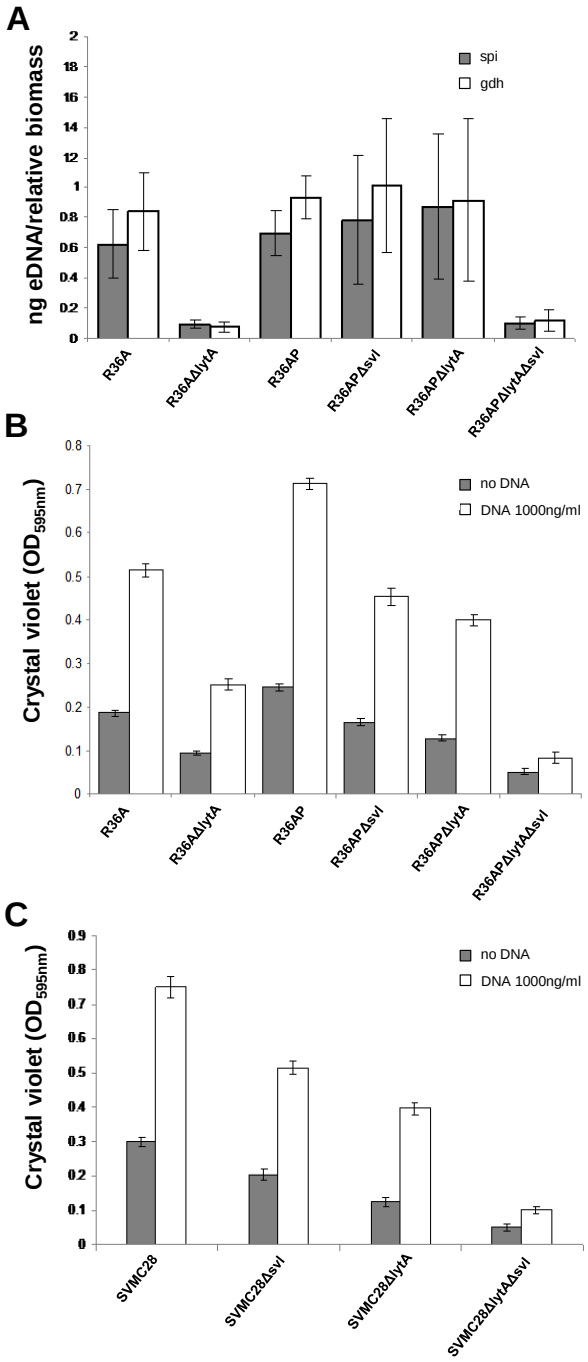

Figure IV.4. eDNA quantification and DNA impact on biofilm mass. (**A**) Extracellular DNA was isolated from the biofilm matrices of R36A, R36AΔ*lytA*, R36AP, R36APΔ*svl*, R36APΔ*lytA* and R36APΔ*lytA*Δ*svl* and quantitative real-time PCR of two chromosomal genes, *spi* and *gdh*, was done. The relative biomass was quantified at OD_{595nm} and the eDNA measurements were normalized to total biofilm mass. (**B**) The effect of salmon sperm DNA (1000 ng/ml) added from seeding on biofilm biomass at 24 h was tested. R36APΔ*svl*, R36APΔ*lytA*, R36AΔ*lytA* and R36APΔ*lytA*Δ*svl* are mutants in which the phage lysin (Svl), the bacterial autolysin (LytA) or both were deleted. (**C**) The same experiments described in panel B were done with the encapsulated wild-type host of phage SV1, strain SVMC28, and its mutants. SVMC28Δ*svl*, SVMC28Δ*lytA* and SVMC28Δ*lytA*Δ*svl* are mutants in which the phage lysin (Svl), the bacterial autolysin (LytA) or both were deleted. In all panels, the results are an average of 9 independent replicates and error bars represent 95% confidence intervals for the sample mean.

To test if the presence of a capsular polysaccharide could influence our results, we characterized the behavior of strain SVMC28 and its mutants in both phage and bacterial lysins. SVMC28 is an encapsulated strain and the natural host of the SV1 phage. The results obtained were superimposable to those of strain R36AP and its mutants, with the same relative biomass produced by the parental strain and its mutants in the absence of DNA and the same effect seen upon DNA addition (Fig.IV.4C). Again, the number of viable cells was consistent with the biomass quantification (Fig.IV.S1B). This indicates that our observations were reproducible in different genetic backgrounds and, more importantly, that the capsule did not qualitatively alter our conclusions. Overall, our results indicate that the release of eDNA through controlled lytic events is a key factor for biofilm formation in *S. pneumoniae* and that lysogenic phages are important adjuvants for its incorporation in the biofilm matrix independently of the presence of a capsular polysaccharide.

5. DISCUSSION

Prophages are extremely common among *S. pneumoniae* isolates causing infections in humans [24]. The lysogenic lifestyle results in the establishment of the phage genome inside the bacterial host where it can remain in a dormant state replicating together with the bacterial chromosome. An important feature is the possible transition from the repressed lysogenic state to lytic development that ultimately leads to host cell death and release of the newly produced phage particles. Prophage induction can occur spontaneously in a fraction of the lysogenic bacterial population or massively upon external stimuli [26,27].

Here we investigated the impact of lysogeny in *S. pneumoniae* biofilm formation exploring its role in the early development of these structures. Our data provided evidence that prophage carriage had a positive impact on pneumococcal biofilm formation through spontaneous induction of the lytic cycle. Phage induction results in the death of their bacterial hosts, however we showed that this phage-mediated lysis enhances biofilm formation, suggesting that in this context the bacterial population as a whole could benefit from limited prophage induction. Studies on gene expression in biofilms of various species have identified phage genes as overexpressed relative to planktonic growth while other studies showed the existence of lysis inside biofilms and proposed that it could increase biofilm fitness [37-42]. Our results corroborate this previous proposal in the context of *S. pneumoniae* biofilms, clearly identifying the phage activated lytic machinery as a key player in this effect. Interestingly, phage-mediated bacterial lysis within the biofilm has also been described in other bacterial species. However, in contrast to our study, in those cases, phage induction results in the death of a large fraction of the bacterial population and occurs in the later stages of biofilm development [39-42].

The impact of lysogenic phages on pneumococcal populations is still an open question since comparative genomic analysis did not reveal any phage-encoded virulence factors, contrary to other related streptococcal pathogens such as *Streptococcus pyogenes* [43,44]. The observed biofilm potentiating role of lysogenic phages and the proposed importance of these structures in colonization [11] could explain in part the high incidence of lysogeny in *S. pneumoniae* natural populations [24,25]. Furthermore, a high frequency of lysogeny is characteristic of many bacterial pathogens [45] as well as of bacterial populations in the environment [46], raising the possibility that the influence of lysogeny on the ability of pneumococci to form biofilms could be paralleled in other bacterial species.

The mechanism by which spontaneous prophage-mediated cell lysis leads to increased biofilm development was also addressed in this study. We gathered evidence that DNA

released through this process to the extracellular environment contributes to biofilm formation in *S. pneumoniae*. An approximately six-fold increase in eDNA was detected in strains carrying prophages and functional bacterial or phage lysins. These lysogenic strains were also characterized by forming biofilms with a higher biomass and cell viability. This role of eDNA is consistent with previous findings in this species, although in those studies the source of eDNA was not identified [5,23]. We observed that eDNA is not involved in the initial attachment stage, since pretreatment of the plastic substrate with DNA did not increase biofilm formation. In agreement, a high concentration of DNAse I added from the onset of biofilm incubation still allowed bacterial surface attachment and biofilm formation, although in these conditions bacteria failed to form the thick and dense structures observed in the absence of DNase I. To our knowledge, this is the first study of the role of eDNA in initial adhesion of pneumococcal cells to a surface. Although in some bacterial species eDNA plays an important role in this initial step [16,47], similar results to ours were already observed with another Gram-positive bacterium [12]. Being such a complex lifestyle, it is plausible that in different microorganisms the importance of the various mechanisms for biofilm establishment is also different. The factors or substances that promote initial attachment remain to be identified in *S. pneumoniae*. However, eDNA played an important role already in the early stages of biofilm development, since spontaneous phage-induced lysis is detected in the early hours of biofilm establishment and the R36AP lysogen showed a more robust biofilm development at all time points. Accordingly, a mutant lacking the phage lysin produced less biofilm and in a delayed fashion, a behavior that was similar to the mutants lacking the major bacterial autolysin LytA. Both observations are consistent with a possibly slower accumulation of eDNA in the matrix and with an important role of eDNA at various stages of biofilm formation.

Our data indicates that eDNA is an important structural component of *S. pneumoniae* biofilms, ensuring stability of the overall architecture of these structures. Although DNAse I treatment resulted in eDNA degradation with the consequent reduction in biofilm formation, the critical result that definitely establishes this structural role of DNA was the observation that addition of fragmented DNA did not affect biofilm development, whereas intact DNA led to increases in both mass and bacterial viability in biofilms, indicating that the long strands of DNA may allow more intercellular cohesion thereby increasing biofilm stability. These results are supported by studies in other species that have proposed DNA as an essential component of the extracellular polymeric substance that constitutes the biofilm matrix [12,15,16,23,48]. Thus, cell lysis mediated by lysogenic phages influences the matrix composition, thereby contributing to the pneumococcal biofilm structural stability. Since spontaneous phage

induction occurs in different areas of the biofilm, it is expected to contribute significantly to the abundance and widespread localization of eDNA.

In contrast to limited cell lysis due to spontaneous phage induction, massive phage induction in the presence of an external inducing agent could disrupt biofilms drastically, an hypothesis supported by the use of lytic phages as powerful antibiofilm agents active against different microorganisms [49,50]. In fact, preliminary results from our group indicate that mitomycin C phage induction is able to disrupt to a large extent biofilms of lysogenic strains (supplementary data, Fig.IV.S2). If the proportion of induced cells is large, more cells lyse than are contributing to the biofilm resulting in an overall loss of biofilm mass. This is in agreement with the natural biofilm demise mediated by substantial phage induction proposed for some *Pseudomonas aeruginosa* strains that facilitate differentiation and dispersal of biofilm associated bacteria [39,41,42]. The beneficial or detrimental effect of prophage induction on biofilm formation seems to be quantitatively regulated by the proportion of lysogenic bacteria undergoing lytic induction.

In conclusion, we showed that limited activation of prophages into the lytic cycle, thereby promoting host lysis and eDNA release, contributes to enhanced pneumococcal biofilm production. This more efficient biofilm development afforded by lysogenic phages may be an important aspect in the biology of the bacteria since lysogeny is highly prevalent in pneumococci. Our data provided new insights into the factors that influence the formation and maintenance of biofilms whose occurrence and importance *in vivo* is increasingly recognized.

6. ACKNOWLEDGEMENTS

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Conceived and designed the experiments: M.C., M.J.F., F.R.P., J.M-C., M.R. Performed the experiments: M.C., M.J.F. Analyzed the data: M.C., M.J.F., F.R.P., M.R. Wrote the paper: M.C., M.J.F., F.R.P., J.M-C, M.R.

7. SUPPLEMENTARY DATA

The following supplementary data are available for this chapter:

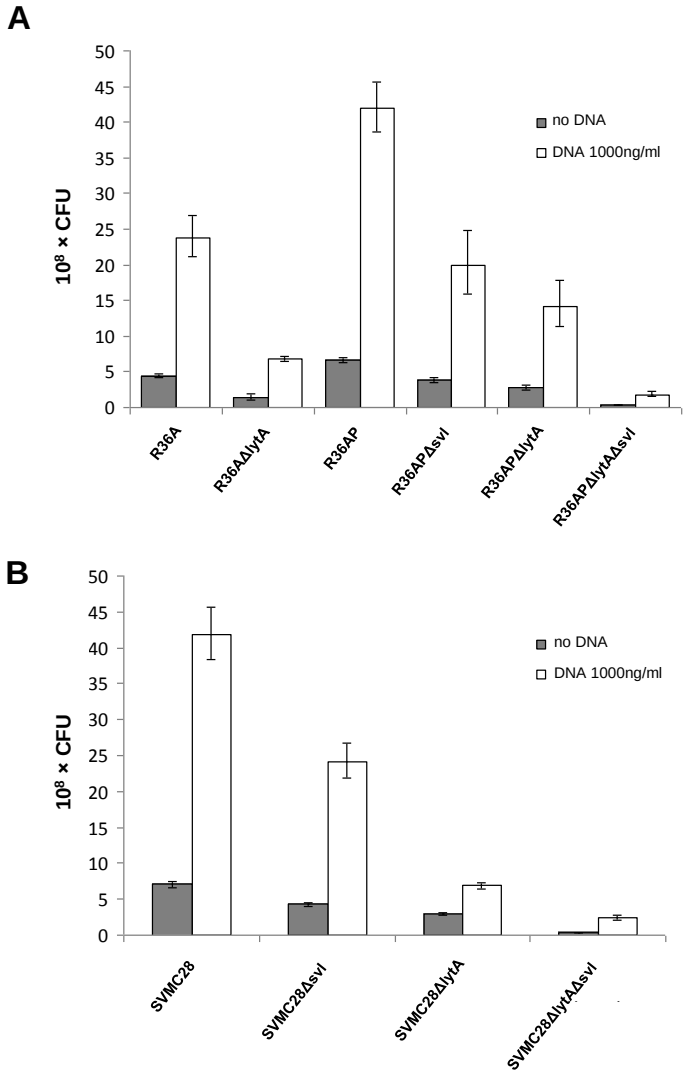

Figure IV.S1. DNA impact on biofilm cell viability. (**A**) The effect of salmon sperm DNA (1000 ng/ml) added from seeding on biofilm cell viability (CFUs) at 24 h was tested on R36A, R36AP and derivative mutants. (**B**) The same experiments described in panel A were done with the encapsulated wild-type host of phage SV1, strain SVMC28, and its mutants. In all panels, the results are an average of 6 independent replicates and error bars represent 95% confidence intervals for the sample mean.

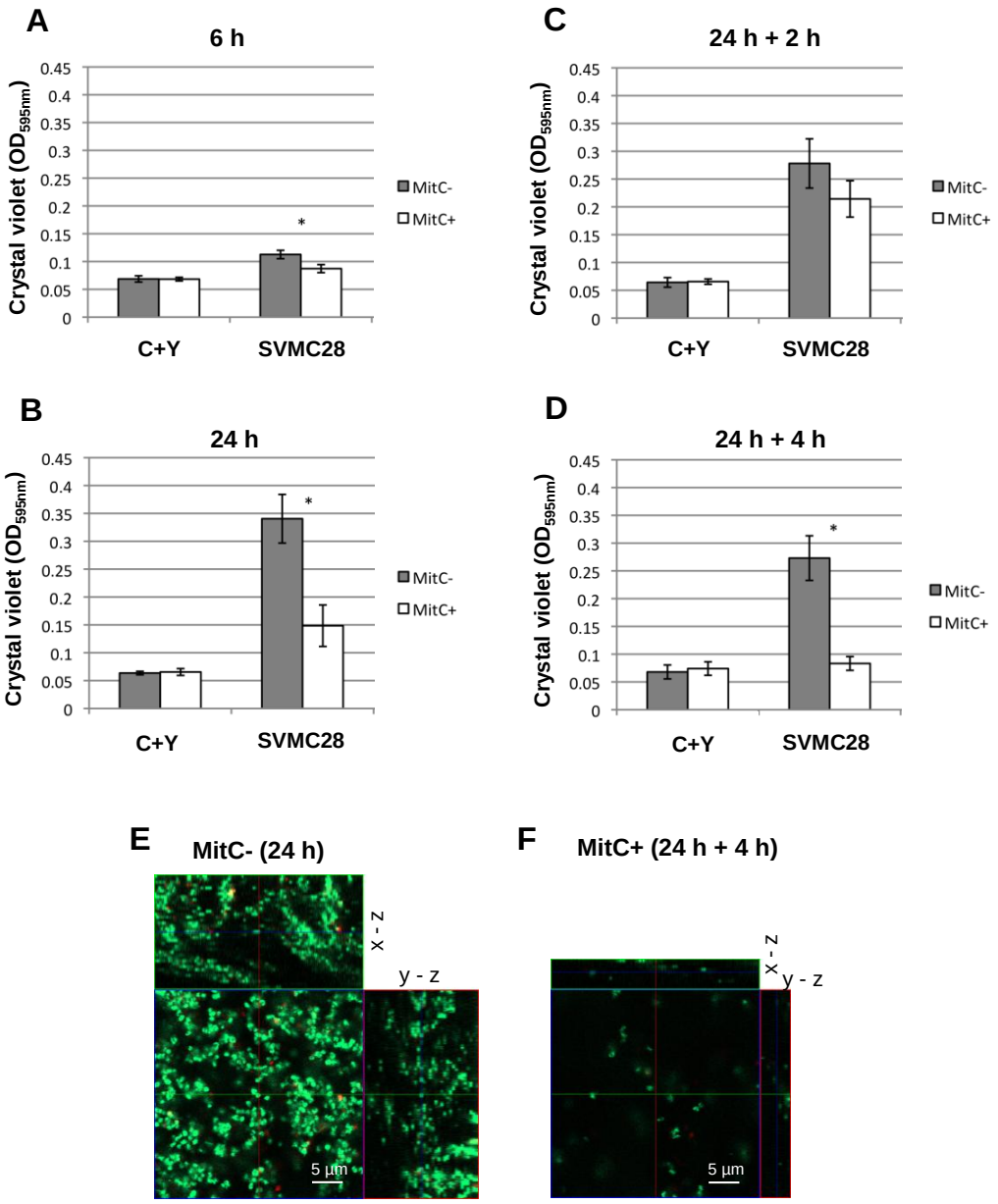

Figure IV.S2. Effect of agent-mediated phage induction in *Streptococcus pneumoniae* biofilm development. (**A** and **B**) SVMC28 wild-type strain was exposed from seeding to mitomycin C (MitC+) to induce the phage lytic cycle at a final concentration of 0.1 µg/ml. A control without MitC was also done (MitC-). Biofilm mass was quantified after 6 h (**A**) and 24 h (**B**) of incubation, as described in material and methods. At both time points, SVMC28 treated with MitC shows a significant decrease on biofilm mass compared to the untreated control. Thus, MitC phage induction at the onset of biofilm formation hindered biofilm development. (**C** and **D**) Biofilms grown for 24 h were exposed to MitC for 2 h (**C**) or 4 h (**D**). After 4 h of incubation with MitC, SVMC28 has a significant decrease on biofilm mass. Thus, MitC phage induction degraded grown biofilms. In all panels, error bars represent 95% confidence intervals for the sample mean. *, $P < 10^{-4}$ as compared with untreated strain; C+Y, control of culture medium (the values were not subtracted to those measured for SVMC28). (**E** and **F**) Confocal laser scanning microscopy images of SVMC28 biofilms were obtained at 24 h of incubation (**E**) and at 24 h of incubation followed by 4 h of MitC exposure (**F**), following the procedure described before. Staining was done with Syto 9/PI and images were acquired at 630x amplification. Live cells internalize only Syto 9 (fluorescing green) whereas dead cells allow the uptake of PI (fluorescing red). The large images are optical sections of top views and the small images to the right and above are optical sections of side views. The depth of the biofilm is indicated by the height of the *z*-stack. The inset scale bar represents 5 µm. In all panels, the results are representative images of 3 independent experiments. In agreement with the results obtained by biomass quantification, treatment with MitC reduces drastically biofilms. Altogether, these results suggest that massive prophage induction, in the presence of an external inducing agent, could disrupt pneumococcal biofilms due to host lysis.

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CHAPTER V

CONCLUSIONS AND FINAL REMARKS

1. CONCLUDING REMARKS

Pneumococcal prophages are extremely frequent among strains associated with infection [1-6] but no role in pathogenesis has so far been attributed to them. A potential contribution may be through bacterial lysis, either by releasing proinflammatory and virulence factors resembling LytA [7-13], or by enhancing biofilm development since the ability to form these structures is apparently important in pneumococcal infection [14-17]. Therefore, this study concentrated on the lysis strategy of *Streptococcus pneumoniae* phages to release their progeny specifically the precise mechanisms involved and its implications on the bacterial host in the context of biofilms.

Concerning the study of the holin-lysin system in pneumococcal phage-induced lysis, a global picture for phage release emerged (Fig.V.1). *S. pneumoniae* phages encode their own lysins which apparently exploit the host Sec system to target the cell wall. In the extracytoplasmic environment, the phage lysins are bound to choline residues and inactive due to the energized membrane. Upon proton motive force (pmf) dissipation provoked by the holin action at the membrane level, lysin activation is accomplished. This in turn, also guarantees the activation of the host autolysin LytA also positioned in the cell wall via choline (Fig.V.1). By exploring the autolysin lytic activity, the pneumococcal phages optimize their exiting strategy maximizing the number of phage particles released. Also, with both lysins already positioned in the cell wall target, this may ensure a quicker hydrolysis of the thicker peptidoglycan of this Gram-positive bacterium allowing a more prompt phage egress. Interestingly, it was observed that DNA damage by mitomycin C (MitC) treatment results in increased *recA* expression (dependent on the development of competence) [18,19] and, in the absence of RecA, does not lead to prophage induction and cell lysis [19]. Also, an overexpression of *lytA* is observed during competence development as it is cotranscribed with *recA* [20-22]. It is therefore tempting to speculate that upon prophage induction higher concentrations of LytA would therefore be at phage disposal.

The establishment of this lysis mechanism was based on the particular lysogenic phage SV1 but a generalization is highly plausible given the presence of the typical holin-lysin cassette in all pneumococcal phages [5,6,23-26], the ubiquitous presence of LytA in *S. pneumoniae* strains, the absence of signal sequences in all phage lysins and the high similarity between lysins of different phages including the presence of a choline binding domain [1,23,27-30]. Our findings that bacterial and phage lysins are under similar physiological control and are both cell surface localized is also coherent with previous reported observations namely the culture lysis only in the stationary phase promoted by the phage lysins Cpl-1 and Hbl, replacing the

autolysin in a *lytA* mutant, despite their constitutive expression as assessed by deoxycholate (DOC)-lysis induction during exponential growth [31].

It was initially assumed that pneumococcal phage lysins depended on holin to gain access to the cell wall [23,24,32,33] similarly to the accepted mechanism operating in the majority of Gram-positive bacteria phages, which are also typically devoid of secretory signal sequences in their lysins [34-36]. Additionally, the observed membrane lesions induced by Cph-1 and Ejh holins were large enough for lysins to pass through [24,26,32]. Our findings, although contradicting this model, are similar to the results reported for phages Cp-1 and EJ-1 [24,26], since holins indeed control phage lysin activation but by membrane deenergization. We also observed that bacterial lysis is prevented in the presence of only the holin or the lysin, although it is induced in the presence of both functions. The holin, but not the lysin, induces membrane permeabilization in conformity with the described holin- but not lysin-induced viability loss. The alleged role of holin permeabilization in lysin transport could not however explain the puzzling observation that the pneumococcal autolysin was able to lyse *Escherichia coli* cells when expressed together with the phage holin [24,26] despite LytA being found in the cell envelope of *E. coli* and its expression in the absence of holin did not result in cell lysis [24,26,37].

In conclusion, pneumococcal phages carry exolysins, operating through the holin-exolysin system, therefore dependent for activity on the holin deenergizing role. This lysis strategy may be more widespread than originally thought since, similarly to what happened in *S. pneumoniae*, it is possible that it might have been overlooked. Interestingly, in Gram-positive bacteria, phage lysins typically have a cell wall binding domain to target them to the cell surface and in some cases a signal sequence seems to be present [35,38-41]. Moreover, some bacterial cell wall hydrolases are unusually deprived of known signal sequences but are still translocated across the membrane (via SecA2 system) [42], and membrane pmf dissipation can trigger lysis in a few bacteria [43,44], which would imply the same influence on phage lysins. It should be mentioned however, that one particularity of *S. pneumoniae* is the high similarity and absence of export signals in both phage lysins and the bacterial autolysin [23].

Research on bacterial autolysins has been stimulated by a desire to determine the mechanisms by which these potentially lethal enzymes are controlled. In this regard, the study of *S. pneumoniae* phage lysis mechanism revealed to be of great value. Indeed, we determined that autolysin LytA is regulated by the membrane pmf, similarly to the phage lysins. However, the exact mechanisms by which loss of membrane energization is a precursor for lysis remain to be determined. The holin-induced membrane deenergization (possibly inducing cell surface perturbations) could be the triggering event of the general disorganization of the lysin-lysin

inhibitor (e.g. the lipoteichoic acid) complex, leading to the uncontrolled action of the lysins or otherwise signal an independent pathway. Another possible explanation relies on pH. The regulation of autolytic enzymes in *Bacillus subtilis*, shown to be dependent on the energized state of the membrane, is proposed to be due to the resulting relatively low pH of the cell wall environment [43,45,46]. Dissipation of pmf would alter the local cell wall pH and, in turn, activate the autolysins [45,46]. Curiously, in *S. pneumoniae*, it was reported that acid stress triggered LytA-mediated lysis even though it was suggested that an intracellular signalling pathway is elicited due to cytoplasmic acidification [47].

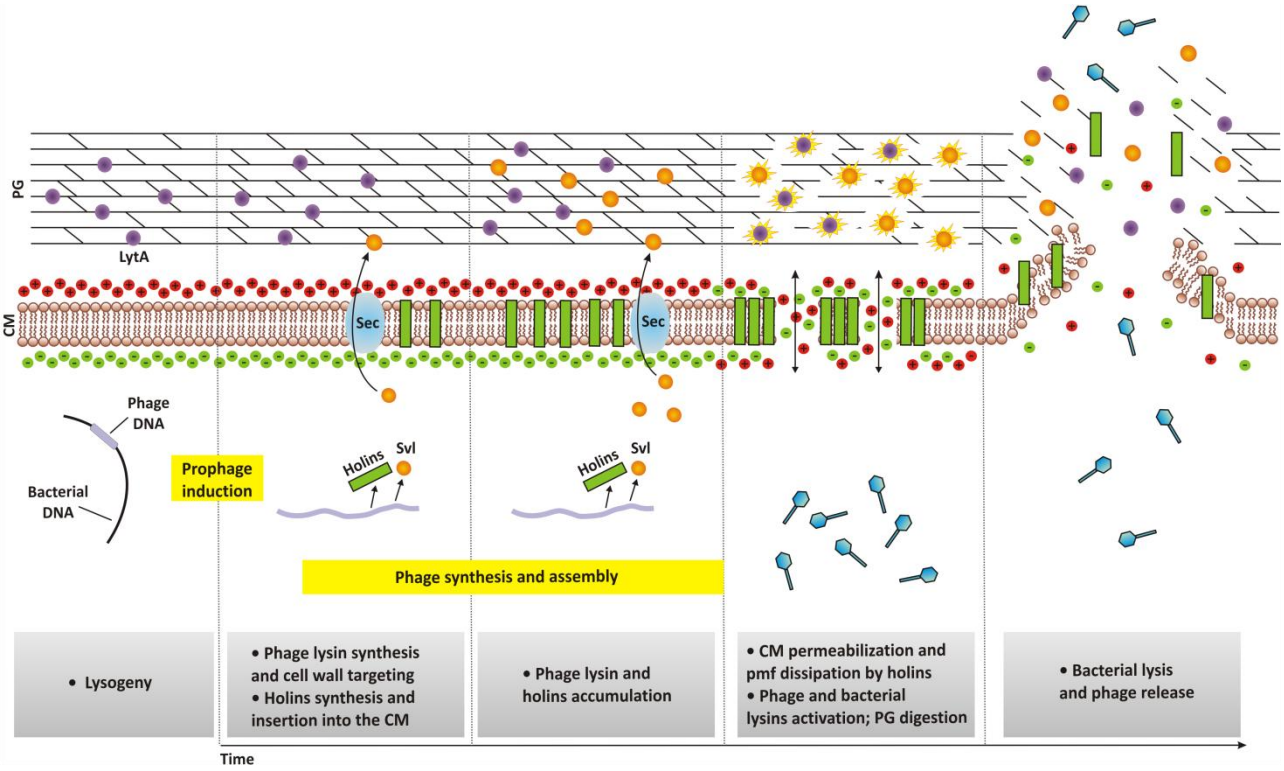

Figure V.1. Proposed model of pneumococcal phage-mediated cell lysis, based on experimental observations with SV1 lysogenic phage. The phage lysin (Svl) is continuously targeted to the cell wall during phage assembly, apparently through the host Sec system, where its activity is regulated by the energized cytoplasmic membrane (CM). Meanwhile, the holins probably accumulate in the CM without affecting the membrane integrity, similarly to what has been proposed for holin Ejh of *S. pneumoniae* phage EJ-1 which accumulates in the CM until it reaches the lesion-forming concentration [32,33]. At the time the holins permeabilize the CM, the induced membrane deenergization results in the activation of the phage lysin and the cell wall localized bacterial autolysin (LytA), which then digest the peptidoglycan (PG) promoting phage release. LytA and phage lysin Svl are coloured in purple and orange, respectively. For simplicity, lysins attachment via teichoic acids is not represented and holins are drawn as single membrane-spanning rectangles in green. Energized membrane is illustrated as "plus" and "minus" charges enclosed in circles.

Furthermore, β-lactamic antibiotics have been shown to induce autolysis and to depolarize the cell membrane potential in *Streptomyces griseus* [48]. Both permeabilization and depolarization of the membrane were also shown to occur when *Staphylococcus aureus*

and *Micrococcus luteus* are treated with amoxicillin [49]. It is known that pneumococci lyse with β-lactams [50] and we observed that membrane deenergization triggers LytA, which is suggestive that these compounds may also induce membrane depolarization in *S. pneumoniae* and thereby activate LytA. This would fit in the Cid model proposed by Morreilon et al. [51], in which the putative holin-like protein Cid would cause injury to the cell membrane when present at large concentrations induced by β-lactams. In the absence of antibiotics, Cid protein at normal physiological concentrations, acting in the membrane, would control the transport of LytA to the cell wall. However, in light of our results, it can be speculated that the loss of membrane integrity would serve to deenergized the membrane rather than allowing the release of large amounts or unregulated forms of LytA. Of interest, the CidA protein of *S. aureus* was actually demonstrated to form membrane lesions similar to holins, to control lysis and to induce an increased sensitivity to penicillin-induced killing, which led to the proposal that this protein may cause the membrane pmf to collapse [52,53]. It should be noted that inhibition of pneumococcal cell wall synthesis by β-lactams apparently promotes the release of lipoteichoic acids suggesting that it destabilizes inhibitor complexes [50], involving or not pmf dissipation. Furthermore, a function of Cid to mediate LytA export could be discarded if LytA follows the Sec pathway like we suggest for the pneumococcal phage lysins.

Remarkably, besides possessing the general Sec system, *S. pneumoniae* apparently also shares the uncommon property of comprising a SecA2-dependent protein secretion [54,55] with some other Gram-positive bacteria including some streptococci [56-60]. Indeed, the *secA2* gene is highly frequent among pneumococci [54] and the detailed analysis of the accessory *secA2* locus in pneumococcal strain TIGR4 revealed that it is very similar to that of other *Streptococcus* species especially *S. gordonii* [54,58], in which some SecA2-dependent proteins were already identified [42,57,59]. It is possible then that in *S. pneumoniae* this could as well represent a specialized transport system that might also export, similar to *Listeria monocytogenes*, a specific subset of unusual proteins lacking known signal sequences including, for instance, some choline binding proteins and even the long-thought cytoplasmic pneumolysin [42,55,61,62]. Thus, as the transport of LytA remains so far unidentified despite several efforts, the hypothesis of the involvement of the accessory SecA2 protein secretion system in lysin transport is important to address.

One biological function of lysis induced by pneumococcal lysogenic phages, demonstrated in the present study, is the enhancement of biofilm formation. The entire bacterial population within the biofilm benefits from the spontaneous prophage induction in a subfraction of cells. These random lytic events contribute to early development of *S. pneumoniae* biofilms by causing release of genomic DNA, which ultimately becomes a critical component of the biofilm

matrix. A structural role of extracellular DNA (eDNA) was definitely confirmed in agreement with the already described eDNA presence in pneumococcal biofilms and its influence in their formation [63,64]. A feature of lysogenic cells is that all are characterized by the presence of a prophage. Therefore, within the biofilm, spontaneous phage induction provides a widespread eDNA source for optimal development of stable biofilms, rather than spatially and temporally confined as proposed for autolytic events [65]. The process of spontaneous induction is not very well understood but it is seems dependent on bacterial physiological conditions [66-68]. Differently from planktonic growth, altered transcriptional profiles associated with phenotypic changes are a characteristic of the complex biofilm form of living [14,69,70] and also vary during biofilm development, as was indeed detected in pneumococcal biofilms [69]. Therefore, it is reasonable to believe that biofilm physiological heterogeneity can play a role in different levels of spontaneous induction and consequently in DNA availability within these structures. It should be mentioned that the fact that few dead cells were detected in the biofilms of wild-type lysogens comparing to the ones of nonlysogens seems to be in agreement with the fast kinetics of the lytic process observed earlier, where intermediary dead cells are present only for a very short time. Indeed, our flow cytometry analysis of MitC-induced wild-type lysogens detected very few permeabilized cells.

Besides the phage positive contribution to pneumococcal biofilm, in this study it was also confirmed that LytA stimulates the formation of these structures as previously described [63]. Since eDNA is important in *S. pneumoniae* biofilms, we explored this relationship further in a phage free context by demonstrating that DNA release from pneumococcal cells is in fact mainly mediated by the autolysin. Elimination of LytA reduced drastically eDNA and DNA addition restored the ability to form robust biofilms in a *lytA* mutant. These results are in agreement with the reported release of eDNA by autolysins in biofilms of other species [71-74]. Because LytA has a lytic activity which is accompanied by DNA release in liquid culture [75-77], it is highly likely that, within the biofilms, LytA activity also results in lysis. How pneumococcal LytA-mediated lysis is limited to a subpopulation of bacteria within the entire biofilm population is a challenging subject to explore.

Although bacterial autolytic events were proposed to exist in biofilms favoring their development [65,71,72,78], the involvement of lysogenic phages in bacterial biofilms has been disregarded. In biofilms of *S. aureus* phage activities were detected but their impact was not evaluated [79]. Despite this, the relevance of phage-mediated lysis has been described in *Pseudomonas aeruginosa* biofilms, where it influences the processes of differentiation and dispersal in already mature biofilms [80,81]. Although in these cases phage induction occurs in a substantial fraction of cells, it is still an advantage for the biofilm life cycle [80,81]. Our

analysis provides a different perspective in the context of *S. pneumoniae* biofilms, namely the impact of the limited lysis intrinsic of the lysogenic state in the early establishment of these structures. In *S. pneumoniae*, this could be of major importance since lysogeny is extremely abundant. Additionally, given the widespread nature of lysogenic phages, it is possible that lysis controlled by them can be as well a key mechanism in biofilm development in other species. Indeed, subsequent studies in *P. aeruginosa* and *Shewanella oneidensis* biofilms support this hypothesis as it was demonstrated, similarly to our findings, a beneficial role for restricted prophage-induced lysis and eDNA release [82,83].

Contrasting with the favorable role of spontaneous fashion of phage induction, massive induction by an external stimulus and consequent substantial lysis seems to destroy to a great extent the biofilms of pneumococcal lysogenic strains. In fact, MitC was able to penetrate the cells encased in the biofilm matrix and disrupted almost completely the biofilms, in agreement with the successful use of lytic phages to eliminate biofilms of different species [84-86]. Interestingly, *S. pneumoniae* phage lytic enzymes, and even the bacterial autolysin LytA, have been shown as efficient weapons in killing pneumococci *in vitro* and *in vivo* [87-92]. Even though the potential of these lysins as antibiofilm agents is little studied, the lysin of phage Cp-1 is likely to prevent biofilm formation in *vivo* [16,17,93]. Furthermore, it was very recently demonstrated their ability to destroy pneumococcal biofilms produced *in vitro*, in spite of not all showing a high disintegrative capacity [94]. This highlights the importance of further investigating the usage of the phage lytic ability.

After exploring the lysis mechanism of *S. pneumoniae* phages and its contribution to biofilms, a question comes to mind especially considering the high prevalence of pneumococcal prophages [1]. What could be the potential role of the lysis mediated by phages in pneumococcal virulence? It can be speculated that in the human host, spontaneous phage induction could favor biofilms potentially contributing to pneumococcal colonization and infection as biofilms have been implicated in both these processes [14,95]. Moreover, independently of pneumococci existence in biofilms, massive phage induction, and to a less extent spontaneous induction, could contribute to virulence in a similar fashion to LytA by releasing cell wall fragments with proinflammatory activity and cytoplasmic virulence factors [7-10]. Interestingly, if virulence factors encoded in pneumococcal prophage genomes are found, prophage induction could increase their expression similarly to what happens in other bacterial species [96]. Actually, prophage-inducing factors are present in the human body including agents produced by human cells such as reactive oxygen species during inflammation in colonization and infection [96,97]. Importantly, although antibiotics are effective at killing pathogens, they could also induce massive phage induction including not only quinolones that

interfere with DNA replication, and therefore are more expected to destabilize the prophage state through SOS response activation, but also β-lactams [98-101]. Although in *S. pneumoniae* the prophage induction by quinolones was not yet studied, it was shown that they also hamper DNA topoisomerases activity [102-104] and seem to induce *recA*, similarly to the DNA damage caused by MitC as mentioned above [18]. Curiously, some quinolones were shown to cause *lytA* overexpression [105], which might be a favorable scenario for phages entering the lytic cycle as hypothesized before. Therefore, the usage of antibiotics could have a negative outcome to the human host as suggested in studies with *S. pneumoniae* where nonbacteriolytic antibiotics (that do not induce LytA activity) reduced release of pneumolysin and reduced the inflammatory host reaction in animal models of infection [12,13].

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