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Mode of Action of Penicillin in Pneumococci: From Inhibition of Cellular Targets to Bacterial Death

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MODE OF ACTION OF PENICILLIN IN PNEUMOCOCCI: FROM INHIBITION OF CELLULAR TARGETS TO BACTERIAL DEATH

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Abstract

While the primary biochemical targets of ß-lactams have been identified, the mechanisms by which inhibition of these targets (the membrane bound penicillin-binding proteins, or PBPs) leads to the irreversible antibacterial effects of these drugs have remained elusive. Treatment of *Streptococcus pneumoniae* with penicillin (or other cell wall inhibitors) results in a rapid sequence of events beginning with a slow-down and brief halt in bacterial growth, followed by rapid and irreversible reduction in the reproductive capacity (killing) of the cells, plus massive degradation (depolymerization and release) of the cell wall sacculus and release of cytoplasmic contents (bacterial lysis). By the end of the 1970s, a large body of observations clearly indicated that all these pathological events observable in penicillin-treated bacteria were secondary, indirect consequences of the inhibition of the primary (enzymatic) targets of penicillin (84). Exactly how and why inhibition of PBPs leads to slow-down and inhibition of growth followed by viability loss and lysis (in some bacteria and under some experimental conditions) has remained poorly understood. However, in pneumococci, the phenomenon of penicillin-induced cell wall degradation and lysis has been shown to result from the (drug-induced) deregulated activity of the pneumococcal major autolytic enzyme, a *N*-acetylmuramyl-L-alanine amidase (referred to as amidase)(80). The concurrence of culture lysis and loss of viability in these organisms has led to the widely held belief that the cause of

cell death was the unregulated (suicidal) activity of bacterial autolysins. On the other hand, in certain bacteria, penicillin can have powerful bactericidal activity without accompanying cell lysis (e.g., pneumococcal mutants with inactivated amidase (*lytA*) gene (80) and group A streptococci), suggesting that mechanisms other than bacterial autolysis may exist for the cidal effect of this antibiotic. We have investigated the existence of such autolysis-independent lethality in pneumococci. The results of these experiments are presented in four Chapters to be summarized next.

Chapter I describes three types of experiments which suggest that only part of the penicillin-induced lethality is due to autolysis by amidase. (i) Suppression of penicillin-induced lysis by specific inhibitors of amidase protects pneumococci only marginally from killing. (ii) Mutants from which the amidase was completely eliminated by insertion-inactivation or deletion of the *lytA* gene are still killed, albeit at a slower rate than the wild-type Lyt^+ strains. (iii) A new mutation (*cid*$^-$), not related to the amidase gene (*lytA*), causing massive reduction in killing was identified and characterized. "Triggering" of the amidase activity by penicillin *in situ* in growing bacteria is significantly reduced in the presence of the novel *cid*$^-$ mutation, indicating that there is a regulatory interaction between the *cid* gene product and the amidase

Chapter II, describes several attempts to characterize the *cid*$^-$ mutation at the molecular level. Shotgun mutagenesis by insertion-inactivation (using either insertion-duplication or

transposon mutagenesis) failed to yield Cid$^-$ mutants, suggesting that null-mutants in the *cid* determinant(s) might not be viable. The possibility that *cid* is an essential gene is supported by the observation that *cid*$^-$ mutants had an extra nutritional requirement, inseparable in genetic crosses from the *cid*$^-$ mutation itself (i.e., growth of *cid*$^-$ mutants in a chemically defined medium required supplementation of the medium with a 500-1000 kD molecular weight molecule present in yeast extract).

Chapter III presents a step-by-step biochemical comparison of the principal penicillin targets of wild-type Cid$^+$ parents and Cid$^-$ pneumococcal mutants, including the penicillin-binding proteins, the major autolysin amidase and the cell wall peptidoglycan. Physiological experiments suggest that *cid*$^-$ mutation is likely to operate at the level of the plasma membrane.

Genetic blocks in the *lytA* (amidase) and *cid* genes can prevent both of the irreversible antibacterial effects of penicillin (lysis and killing) in pneumococci. Yet, these bacteria remain exquisitely sensitive to the drug, but addition of the antibiotic only causes inhibition of growth. The availability of the Cid$^-$ Lyt$^-$ double mutants allowed us to look closer at the mechanism of this reversible growth inhibitory effect of penicillin and Chapter IV describes studies in this direction. These studies show that penicillin treatment of both Cid$^+$ and Cid$^-$ pneumococci results in the shutoff of protein and RNA synthesis, through some as yet unidentified regulatory circuit in the cells. In addition, antibiotic-treated pneumococci

induce and or continue the specific radioactive labeling of a unique 72 kD protein with [^{35}S]-cysteine, even when incorporation of radioactive amino acids in all other protein species has come to a virtual halt. The mechanism of radioactive labeling of the 72 kD protein in response to inhibition of synthesis of bacterial biopolymers is likely to involve addition of [^{35}S]-cysteine to a preexisting protein through peptide bonds. The 72 kD species is also labeled in response to treatment with some other antibiotics, mechanistically unrelated to penicillin, but not during heat-shock. The 72 kD protein might be an "stress factor" implicated in the regulatory circuit mediating metabolic idling during antibiotic treatment.

Taken together, the results presented in this dissertation underline the complexity of the pneumococcal response to treatment with penicillin. Analysis of Lyt$^-$ and Cid$^-$ mutants and double mutants has allowed a glimpse into several distinct pathological processes that are related to antibiotic-induced disintegration, viability loss, while the discovery of the 72 kD "stress factor" may serve as a signal for halt in growth and turning off cellular biopolymer synthesis. The major conclusion of the thesis work is that the primary mechanism of penicillin-induced lethality is not bacterial lysis. It appears that during penicillin treatment of wild-type (Cid$^+$ Lyt$^+$) pneumococci, the bacteria are first killed and then lysed. Antibiotic-induced killing appears to be mediated by an alternative - membrane-related - mechanism, which can be influenced by mutation(s) (*cid*$^-$) independent of the determinant (*lytA*) of the major

autolysin amidase. The interaction between the bacterial autolysis and the *cid* product may be analogous to the model proposed for the interactions between the membrane-active "channel" proteins (holins) and endolysins in bacteriophage-induced lysis of bacteria (57,96).

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Introduction

I. The bacterial challenge to ß-lactam antibiotics - antibiotic resistance and tolerance.

Over 50 years after the introduction of penicillin in human medicine (2), ß-lactam antibiotics remain one of the most important groups of molecules in the armamentarium of antibacterial agents. The key to this success resides in the fact that these compounds specifically inhibit critical steps in the synthesis of the essential bacterial cell wall peptidoglycan, a structure uniquely conserved among eubacteria. This restriction renders ß-lactam antibiotics highly toxic for bacteria synthesizing peptidoglycan, while being remarkably harmless for other organisms. For example, 6 ng of penicillin/ml are sufficient to inhibit bacterial growth of a penicillin-susceptible strain of *Streptococcus pneumoniae*, whereas up to 100,000 ng of penicillin/ml of serum can be achieved during intravenous treatment of animals without any harm for the recipient. The necessary presence of peptidoglycan for the full-blown toxicity of ß-lactam antibiotics is also demonstrated by the fact that bacteria devoid of this particular structure (such as mycoplasma, archaeobacteria and L-forms of bacteria) are insensitive to these compounds.

An additional very important feature of ß-lactam antibiotics is their bactericidal activity. Bacterial killing is an absolute

requirement for successful treatment of infections in conditions of impaired or absent host defense mechanisms. Such infections are not limited to the "classical" bacterial meningitis or endocarditis, in which the infection is confined to anatomic sanctuaries quasi devoid of phagocytes or other cellular host defense mechanisms. They now also encompass a new spectrum of bacterial diseases in the increasing number of patients with immune defects, including both the so-called acquired immune deficiency syndrome (AIDS) as well as patients with other underlying conditions such as anticancer chemotherapy.

Not surprisingly, during the past decades bacteria have evolved a number of sophisticated mechanisms to escape the bacteriostatic and/or bactericidal effect of ß-lactam antibiotics. These can be broadly separated in two classes: (i) antibiotic resistance and (ii) antibiotic tolerance. Resistant bacteria are characterized by the fact that they can continue to grow in the presence of concentrations of antibiotic that may be far above the threshold that inhibits multiplication of most (i.e., the susceptible) members of the same species, the minimal inhibitory concentration, or MIC. Nevertheless, above this new - elevated - threshold concentration, resistant bacteria again respond to the killing (and lytic) effects of the drug. Tolerant bacteria, in contrast, retain the MIC value characteristic of susceptible strains, but become dramatically altered in their response to drug-induced killing and/or lysis - i.e., they are

able to "survive" drug treatment (29,49,57,85,86)[1].

The antibiotic pressure operating in the clinical environment selects for both tolerant and resistant bacteria (49,58). In such an environment, antibiotic tolerance, i.e., the ability to survive antibiotic treatment, is likely to provide bacteria with a tremendous advantage to equip themselves with resistance genes, either by spontaneous mutations or by DNA exchange. Therefore, in view of the contemporary alarming spread of antibiotic resistance to virtually all known antibiotics (64), the potential importance of antibiotic tolerance is evident. Hence, it is of the utmost importance to understand the mechanism(s) and the nature of the cidal damage(s) inflicted by ß-lactam antibiotics to the bacterial cell.

In the following, I shall briefly overview our current perception of the mode of action of ß-lactam antibiotics and the physiological consequences of inactivation of their specific targets, the membrane-bound penicillin-binding proteins (PBPs). I shall also present evidence arising from both natural and laboratory isolates of bacteria which suggest that antibiotic-induced bacterial lysis might not be, as widely believed, the

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[1]Tolerance can either result from "survival" mutations (genotypic tolerance) or can be naturally expressed in conditions of slow bacterial growth (phenotypic tolerance) (85). In contrast to the latter, genotypic tolerance is expressed during the whole cycle of bacterial growth and is transmitted both vertically to the bacterial progeny and horizontally to other cells by DNA transfer (57). Mutations that confer antibiotic tolerance or resistance are mechanistically and genetically independent (49,57).

major bactericidal lesion produced by penicillin treatment in pneumococci.

II. The primary bacterial targets of ß-lactam antibiotics: membrane-bound penicillin-binding proteins (PBPs)

Penicillin-binding proteins (PBPs) are the primary bacterial targets of ß-lactam antibiotics. They are membrane-bound enzymes which are essential for the assembly of the cell wall peptidoglycan. PBPs have been the subject of extensive research and have been reviewed in the literature (13,23,76,79). The following overview is a non-exhaustive summary of the current understanding of the PBP-ß-lactam interaction, to the extent that is relevant for the subject of this presentation.

(i) The functional anatomy of penicillin-binding proteins: PBPs are members of a larger family of so-called penicillin-interacting molecules, which share the ability to bind penicillin and catalyze the rupture of the lactam amide bond and transfer the penicilloyl moiety to an essential serine, forming a serine-ester-linked acyl derivative (see generic formula [1] and figure 1 on p. 6 and 8 resp.). Each bacterial strain contains a specific set of PBPs, the number and molecular size of which are highly conserved and may even be used for taxonomic purposes. Depending on the organism, a bacterium may contain up to 2,000 PBP molecules. In a given strain, PBPs of different molecular sizes

are numbered from the largest to the smallest molecule.

High molecular weight PBPs (e.g., PBP 1 to 3 in *E. coli*) are believed to be important enzymes that catalyze the final stages of peptidoglycan synthesis. Some of these proteins (but perhaps not all) are bifunctional enzymes that catalyze both a penicillin-insensitive peptidoglycan transglycosylase and a penicillin-sensitive transpeptidase activity (63A) and are anchored to the membrane through a hydrophobic N-terminal segment (76, 76A)[2]. In contrast, known PBPs of low molecular weight (e.g., PBP 5 of *E. coli*) are anchored to the membrane through a carboxy-terminal hydrophobic segment, and contain only a "peptidase" extracellular domain (for review see 23 and 76).

The immediate precursors of peptidoglycan synthesis are believed to be bactoprenol-pyrophosphate-linked muropeptides: (N-acetylglucosaminyl-N-acetylmuramyl)-L-Ala-γ-D-Glu-L-Xaa-D-Ala-D-Ala pentapeptide units[3]. The lipid-linked disaccharide pentapeptide itself is the end product of a multistep cytoplasmic and membrane-located biosynthetic pathway. The completed muropeptide molecules are translocated to the outer face of the plasma membrane for final peptidoglycan assembly. This step presumably requires both a glycan chain elongation and an interpeptide crosslinking machinery, a function which is provided

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[2]PBP 3 is an exception which is lipid-linked to the membrane through a cysteinyl residue.

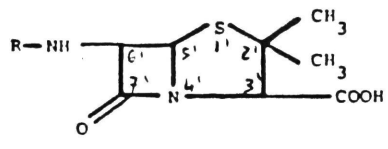
[3]Depending on the bacterial species, L-Xaa is a diamino acid whose ω-amino group is either free or substituted by additional amino acids.

by the PBPs (13,23).

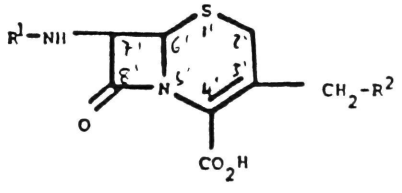
(ii) The mechanism of PBP-inhibition by penicillin: Penicillin interferes with the "transpeptidase" domain of the PBPs. Naturally occurring transpeptidation (which is inhibited by penicillin) is a 2-step reaction (see figure 1, p. 8). First, the terminal D-Ala-D-Ala peptide bond of the of the precursor is split through the nucleophilic attack of its carbonyl group by a conserved serine residue of the enzyme (PBPs are functional analogs of serine proteases). The terminal D-alanine leaving group is released to the solvent and an acyl intermediate is formed between the serine residue of the enzyme and the penultimate D-Ala residue of the precursor (left panel in figure 1, p. 8). Second, the enzyme is freed by the transfer of the latter bond to a free amino group (acceptor) in the stem peptide of the nascent peptidoglycan (not shown in figure 1).

The core of the ß-lactam molecule (i.e., the ß-lactam ring plus the C3′ residue for penicillins and the C4′ residue for cephalosporins; see generic formula [1]) is a steric analog of the ubiquitous D-Ala-D-Ala terminal of cell wall precursors disaccharide-pentapeptides (see left panel in figure 1, p. 8).

[1]



penicillin



cephalosporin

Therefore, penicillin binds to PBPs and undergoes nucleophilic attack at its C7′ carbonyl group in a manner analogous to the D-Ala-D-Ala of the disaccharide-pentapeptide cell wall precursor (see figure 1, p. 8). However, because the scissile bond of penicillin is cyclic, the leaving group of the molecule remains in place, resulting in the incapacity of the enzyme both to transfer the bond to an acceptor or to undergo solvolysis (right panel in figure 1). Therefore, penicillin acts as mechanism-based (suicide) inhibitor of the enzyme (13,23,43,67).[4]

The reaction obeys the following kinetics:

$$[2]\quad E + D \underset{}{\overset{K}{\rightleftharpoons}} E\text{-}D \xrightarrow{k_2} ED \xrightarrow{k_3} E + P$$

where E is the PBP; D is the ß-lactam molecule; E-D is the Michaelis complex; ED is the penicilloyl acyl enzyme; P is the reaction product (penicilloic acid); K is the dissociation constant; and k_2 and k_3 are the first order rate constants for acylation and deacylation respectively (13,79). In most PBPs, k_2 is big and k_3 is small. Therefore, binding of penicillin results in prolonged inhibition of the enzyme.

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[4]Since the original proposition of this model by Tipper and Strominger in 1968 (77), it has gained experimental support and was recently confirmed by co-crystallization of penicillin-interactive proteins with beta-lactam molecules (43,67).

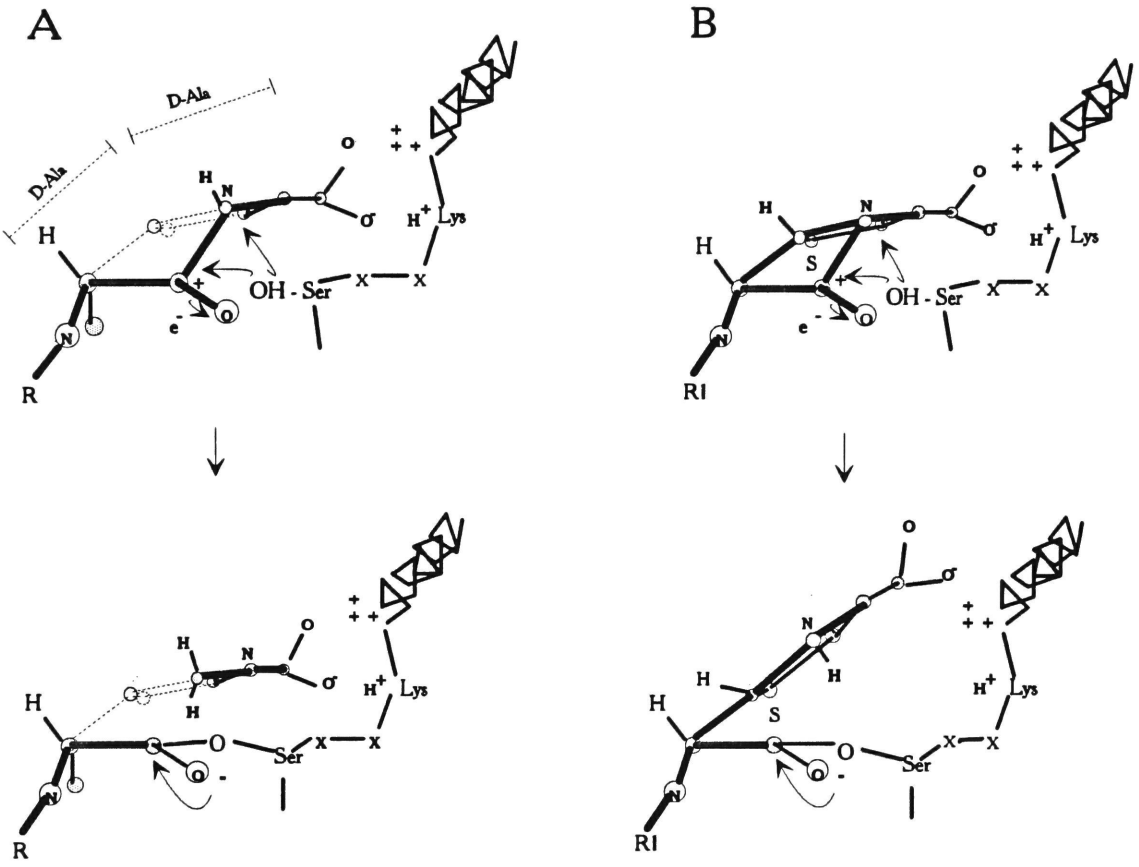

Figure 1: Acylation of penicillin-interactive proteins by either the D-Ala-D-Ala terminal residues of normal cell wall precursors (A), or penicillin (B). In panel A, R represents the proximal portion of the precursor (a disaccharide tripeptide, see text) and the dashed lines depict a virtual molecule of penicillin superimposed onto the D-Ala-D-Ala for comparison. The bottom of panel B shows that after acylation of the enzyme, the leaving group of the β-lactam molecule is not released to the medium, due to the cyclic nature of the scissile bond. The backbone of the protein represents the active site of the enzyme with the Ser–x-x-Lys conserved motif followed by an α-helix which helps maintain a positively charged environment around the carboxyl group of the penicillin molecule.

(iii) The family of penicillin-interactive enzymes: As mentioned above, PBPs belong to a larger (super) family of penicillin-interactive proteins (23). Among these, another important membrane-bound protein (BLAR) is a penicillin sensor which is part of a signal transduction system for the induction of penicillinase secretion in *Bacillus licheniformis* (41). Most of the other members of this family are soluble ß-lactamases. These penicillin-inactivating enzymes are believed to have evolved divergently from PBPs to specialize in detoxification of ß-lactam molecules (42). The nature of the reaction between ß-lactamases and penicillin is identical to that shown for the PBPs, except for the fact that the value of the k_3 constant is much higher. It is thought that ß-lactamases have acquired the ability to hydrolyze the penicilloyl-enzyme bond with H_2O, probably by altering the conformation of the enzyme in order to provide a pocket to accept a water molecule. In the process, they have lost their transpeptidase activity.

Bacteria (particularly gram-negative organisms) have engineered a great variety of ß-lactamases, which can hydrolyze virtually all the available ß-lactam molecules. Moreover, antibiotic pressure from new ß-lactam derivatives selects for mutations and remodeling of existing ß-lactamases to adapt to the new antibiotic molecule. The emergence of new ß-lactamases (as extended spectrum ß-lactamases) produced by ß-lactam resistant bacteria constitute a major challenge to the chemist involved with the design of new effective ß-lactam compounds.

Penicillin-interacting enzymes are classified both by their functional anatomy (i.e., low molecular weight PBPs; high molecular weight PBPs; and ß-lactamases) and according to their primary structure. However, divergence in primary structures are overcome by similarities in the tertiary structure of the proteins. In fact, all penicillin-interactive proteins contain four "signature" motifs of amino acids that may be brought within the enzyme's active center by adequate folding of the polypeptides. These signatures are (i) a tetrad active site serine-x-x-lysine (see also figure 1, p. 8), (ii) a triad serine or lysine-x-asparagine, (iii) a peptide segment (Asp/Glu) that contains two dicarboxylic amino acids and (iv), a triad lysine, histidine-threonine or serine-glycine. A detailed discussion of the biochemical interactions between these residues and the ß-lactam molecules is beyond the scope of this introduction (23,40).

III. The journey of the extracellular antibiotic to its membrane target PBPs

When penicillin is added to a bacterial culture, it has to cross a variety of chemical and physical barriers before it can reach and inhibit its specific targets, the membrane-bound PBPs. The most important physical barrier (in gram-negative bacteria) is the outer membrane (OM). ß-lactams traverse the OM through so-called porin molecules, which provide hydrophilic channels to the

periplasmic space. The bacteria can alter both the pore size and/or the number of the porin molecules, thus interfering with the access of the antibiotic to the periplasm (28,65). Gram-positive bacteria are devoid of OM. However, both gram-positive and gram negative bacteria may produce poly-anionic exopolysaccharides which may also hinder the diffusion of antibiotics to some extent (see 3A). A second obstacle faced by the antibiotic is the presence of specific ß-lactamases - secreted or present in the periplasmic space - which may inactivate the drug before it has reached its targets. As mentioned above, production of ß-lactamases is one of the powerful mechanisms of resistance found both in gram-negative and gram-positive bacteria (44,70)[5]. Eventually, the efficacy of the drug will depend on its ability to overcome these bacterial obstacles and to bind to and inactivate its bacterial targets, the PBPs.

These main steps of the travel of ß-lactam molecules to their targets are also the major variables employed by the bacteria to modulate their resistance to these drugs. Under selective antibiotic pressure, bacteria may alter their OM porins (gram-negative bacteria), produce ß-lactamases (qualitatively new and/or larger quantities) or, reduce the affinity of PBPs for the

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[5] Sensitivity to ß-lactamases can be overcome, to some extent, by adequate modifications in the ß-lactam molecules. This may result in resistance to hydrolysis by the enzyme or even inactivation of the ß-lactamase. Clavulanic acid and sulbactam are examples of the latter compounds (44,70).

antibiotic. These mechanisms of resistance may arise independently, but may synergistically coexist in a single organism. Synergistic resistance is well illustrated by methicillin resistant *Staphylococcus aureus* (MRSA), most strains of which carry both ß-lactamases as well as altered (reduced) affinity PBPs (8,31,53)[6].

IV. Physiological consequences of the inhibition of bacterial PBPs by penicillin.

(i) Halt of bacterial growth. The preceding sections have overviewed the mechanisms by which ß-lactam molecules inhibit specific bacterial targets, the PBPs. Not surprisingly, blockage of these essential cell wall active enzymes results in inhibition and/or profound alteration of peptidoglycan synthesis (23,79). Since bacteria have usually several different types of PBPs, it

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[6] Shortly after the introduction of penicillin in medicine (in the early forties), *Staphylococcus aureus* managed to acquire a penicillinase-encoding plasmid that rendered these organisms resistant to this drug. Twenty years later, shortly after the introduction of the penicillinase-resistant ß-lactam methicillin, methicillin-resistant *Staphylococcus aureus* (MRSA) arose (31). This second mechanism of resistance was not mediated by a new type of ß-lactamase, but by the acquisition of a new, additional PBP (referred to as PBP 2A; (8,53)) with very low binding affinity for most ß-lactam antibiotics. This dual mechanism of resistance (i.e., production of both ß-lactamases and low affinity PBPs) has transformed these organisms into a public health hazard. First, there are very few therapeutic alternatives available to treat severe infections due to MRSA. Second, owing to their double resistance mechanism, MRSA tend to be selected among other (less pathogenic) organisms during ß-lactam treatment of unrelated infections.

was assumed that each of them might perform complementary tasks, the coordination of which resulted in the assembly of a "normal" bacterial sacculus. This assumption was confirmed in *E. coli*, in which the function of specific PBPs was inhibited either by treatment with selectively binding drugs, or by construction of appropriate mutants. It was observed, for instance, that (i) inhibition of PBP 1A together with 1B of *E. coli* was lethal (52B,52C), (ii) inhibition of PBP 2 interfered with elongation (resulting in spherical cells) (76B) and (iii), inhibition of PBP 3 interfered with septation (resulting in elongated bacterial filaments)(76B). On the other hand, PBPs 4, 5 and 6 were apparently dispensable for normal growth under the conditions of *in vitro* cultivation (52D,76C). While selective inhibition of one or the other of the essential PBPs had distinct morphological consequences, they all had a common eventual result: a halt in bacterial growth.

(ii) The irreversible effects of ß-lactam antibiotics: lysis. In an original model of "unbalanced growth", it was assumed that inhibition of transpeptidation (but not transglycosylation) by penicillin resulted in a weakened cell wall, which eventually was disrupted by the increased turgor pressure resulting from the uninhibited increase in the cytoplasmic mass of the bacterium (i.e., combined osmotic and mechanical pressure - from within). As an ultimate result of such unbalanced growth, cell wall integrity was lost, resulting in both lysis and death of the

organism. However, this model had to be revised in the light of two types of later experiments.

First, it was shown that bacterial lysis was mediated by specific (non-penicillin interactive) peptidoglycan (or murein) hydrolases (referred to as autolysins) that became deregulated or "triggered" during treatment of bacteria with ß-lactam antibiotics or other cell wall inhibitors (84). This was originally demonstrated in pneumococci in which the activity of the major autolysin was blocked by physiological intervention or by mutations introduced into the structural gene (*lytA*) of the major autolytic enzyme amidase (a N-acetylmuramyl-L-alanine amidase)(80). When compared to the parent strain (which underwent rapid and extensive killing and lysis during penicillin treatment), the lysis defective mutant (Lyt^-) was not lysed and was killed more slowly by the antibiotic. This new phenotype, for which the term "antibiotic tolerance" was coined (80), was not due to altered PBPs or cell wall structure, but was clearly related to the suppressed amidase activity.

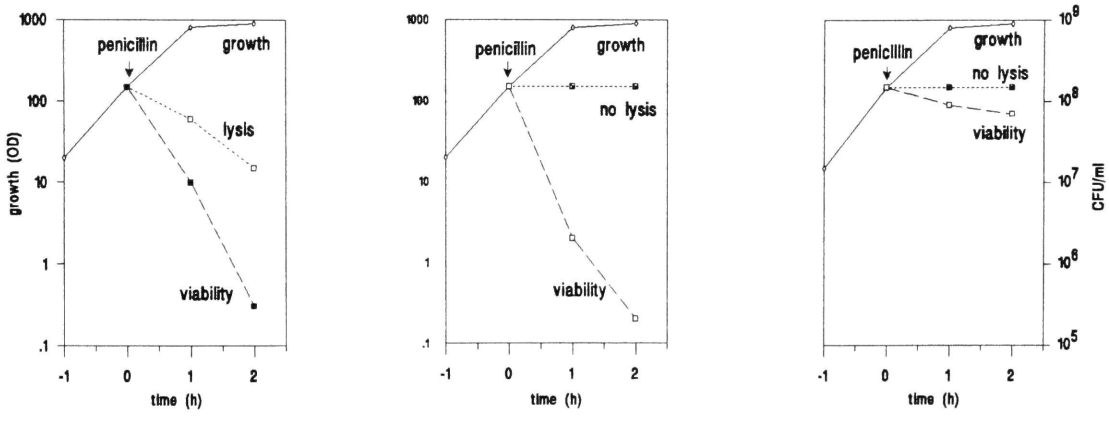
A second type of experimentation with a tolerant strain of another bacterium - *Streptococcus mutans* - suggested that the irreversible effect of penicillin (i.e., bacterial killing and cell lysis) resulted from more complicated interactions than "mere" inhibition of the wall synthesis followed by osmotic rupture. In these experiments, when bacteria were treated with high concentrations of penicillin, inhibition of bacterial growth was paralleled by inhibition of biopolymer synthesis, including

peptidoglycan, proteins, RNA and DNA as well (54,63), suggesting that inhibition of cell wall synthesis actually initiates a coordinate shutoff (rather than continued unbalanced synthesis) of most bacterial polymers. It has been proposed that the primary penicillin targets were only indirectly involved in the irreversible effect of the drug (84).

(iii) The bactericidal mechanism(s) of penicillin: In fast-lysing organisms, such as pneumococci or *E. coli*, the concurrence of rapid bacterial lysis and loss of viable cells (i.e., loss of colony forming units of CFUs) in the culture has led to the widely-held belief that the cause of penicillin-induced death was the "suicidal" (unregulated) activity of autolytic enzymes. On the other hand, it is also known that in certain species of bacteria, penicillin can have powerful bactericidal activity without accompanying autolysis (29,34,55). Examples of the extreme situations are presented in figure 2 (p. 16). On the left part, wild type *Streptococcus pneumoniae* is rapidly lysed and killed by treatment with penicillin. On the right part, *S. sanguis* is neither lysed nor killed by the antibiotic. In the middle, group A Streptococcus is typically resistant to penicillin-induced lysis, while being highly sensitive to the cidal effect of the drug. In this latter case bacteria have lost the ability to divide and form colonies (i.e., lost viability) in the absence of major physical damage to cell structure.

The notion that penicillin-induced killing of bacteria is a

S. pneumoniae	*S. pyogenes* (group A)	*S. sanguis* (group H)



Lysis:	**yes**	**no**	**no**
Killing:	**yes**	**yes**	**no**

Figure 2: **Schematic representation of different responses to penicillin treatment in three species of streptococci (modified from Horne, D., Tomasz, A. 1977. Antimicrob. Agents and Chemother. 11:888-896). Growth of the cultures was measured by the increase in optical density (OD). Penicillin (10x the MIC) was added (arrows) to cultures in the mid-logarithmic phase of growth. Plain lines indicates the OD of untreated control cultures, dotted lines the OD of penicillin-treated cultures and dashed lines the viable CFU/ml, as determined by plating aliquots of the cultures on penicillinase-containing blood-agar plates.**

consequence of osmotic explosion was the logical conclusion of original experiments with spheroplasts of *E. coli* K-12 (47). Penicillin-treated bacteria suspended in an osmotically protective medium generated stable spheroplasts that could revert to rod-shaped cells and resume growth after removal of the antibiotic from the broth. Control bacteria suspended in non-protective medium lysed and lost viability. Osmotic lysis may indeed have been the ultimate cause of viability loss in these particular experiments. However, the interaction between penicillin and intact bacterial cells is clearly a much more complicated process. For instance, while ampicillin-induced spheroplasts of *Proteus mirabilis* did not lose optical density over time, viable counts dropped by several log units during antibiotic treatment (25). In these experiments, the lethal lesion was likely to be different from the mere osmotic explosion of the cells.

Another set of intriguing experiments was reported by Gutmann et al (27). They showed that penicillin-treated cultures of group A streptococci did not lyse, as measured by the absence of (i) loss of optical density of the culture, (ii) morphological damage detectable by electron microscopy and (iii) leakage of radiolabeled proteins. Yet, rapid loss of viability was observed when dilutions of the cultures were plated on nutrient blood agar plates (BAP). However, when plated in parallel on BAP supplemented with high salt plus sucrose, a substantial proportion of colony forming units (CFU) could be rescued. This

suggested the existence of some minimum, yet lethal, penicillin-induced alteration that could be reversed (healed?) by altering either the ionic strength or the osmolarity of the medium. This phenomenon remains unexplained, but demonstrates the absence of major physical damage of the bacteria in this type of penicillin-induced bacterial death.

(iv) The non-lytic bacterial death: There are a number of conditions or agents that decrease bacterial lysis but only marginally (if at all) reduce cell death. These include, for instance, treatment of pneumococci with a variety of specific autolysin inhibitors (57), or overexpression of heat shock proteins in *E. coli* (68,95). Moreover, most pneumococcal (88)[7] and staphylococcal (Oshida, personal communication) isolates bearing regulatory mutations in the autolytic system have been found to be "normally" killed by ß-lactam antibiotics, in spite of the fact that they were only very slowly lysed by the drug. Even pneumococcal mutants with a completely inactivated amidase gene (*lytA* deletion (57,80)) underwent substantial residual killing, in spite of the absence of cell wall degradation and lysis during penicillin treatment. Finally, if the major autolysin amidase was a limiting factor of penicillin-induced

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[7]Despite of greatly diminished rates of penicillin-induced lysis, all of 25 penicillin-resistant (MIC $\geq$ 0.1 ug of penicillin/ml) clinical isolates of pneumococci were killed in a similar way as the lysis-prone wild type control strain when exposed to 10x the MIC of penicillin (data not presented and (88)).

killing, *lytA* mutants should also have emerged from penicillin selection applied in the laboratory or in the clinical setting (49,58,81)[8]. Taken together, these observations suggest that lysis might not be the primary killing mechanism in these conditions.

(v) The search for the killing target: Taken together, these experiments raise the challenge to better define the lethal lesion inflicted by penicillin to susceptible bacteria. However, since ß-lactam toxicity is a multi-level problem for bacteria, there might also be several killing targets, which might depend on both the type of organism and the bacterial environment. For instance, while penicillin-treated wall-less protoplasts (which may contain PBPs (48)) can synthesize proteins and increase mass, they usually cannot divide and give raise to colonies in the presence of the drug. Moreover, L-forms of bacteria are not inhibited by ß-lactams. These observations indicate that the toxicity of the drug might not be directly mediated by blockage of the primary penicillin targets (the PBPs) and suggest that some insoluble cell wall precursors or metabolites are necessary for the full-blown effect of the antibiotic (i.e., growth inhibition and killing). In addition, penicillin-treatment of tolerant bacteria results in the shutoff of biopolymer synthesis suggesting that the cells may respond to inhibition of cell wall

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[8]The *lytA* gene product (amidase) is dispensable for the bacterium (81).

synthesis by a coordinated turn-off of metabolic activities in the bacterium, the mechanism of which is unknown (54,63). Finally, penicillin-treatment of gram positive bacteria is also accompanied by shedding (or secretion) of membrane-bound molecules such as the lipoteichoic acids, which are also involved in the regulation of autolytic enzymes in these organisms (3,67A,82). This may also indicate the existence of antibiotic-induced alterations of the plasma membrane during drug treatment (82). All these different levels must be taken into account in the search for the cause of bacterial death that is not accompanied by lysis of the cells.

V. Outline of the thesis

The experiments to be described in the following chapters are an attempt to define the mechanism(s) of penicillin-induced killing in *Streptococcus pneumoniae*. This organism was chosen as a model for the following reasons. First, wild type pneumococci are exquisitely sensitive to penicillin-induced killing and lysis, which places their phenotype at the left extreme of figure 2 (p. 16), corresponding to what is considered as a classical response to penicillin treatment. Second, in recent years, clinical isolates of pneumococci have escaped the classic phenotype to become penicillin-resistant and sometimes also tolerant to the drug (figure 2 middle and right, p. 16)(49). Finally mutants which were altered in both their killing and

lytic response to penicillin treatment were also isolated in the laboratory (57,58,80).

In the first chapter, I present physiological and genetic evidence for the existence of an autolysis-independent mechanism of penicillin-induced killing in pneumococci. I describe the identification of a new mutation (*cid*$^-$), which reduces penicillin-induced killing in these organisms. The second chapter describes a series of experiments aimed at cloning the so-called *cid*$^-$ determinant. The third chapter is a step-by-step biochemical and physiological comparison of wild type parents and *cid*$^-$ mutants of pneumococci, aimed at defining the level at which the *cid*$^-$ mutation might operate to provide protection against penicillin-induced killing. Finally, in the fourth chapter, I present evidence for a regulated shutoff of RNA and protein synthesis initiated by inhibition of cell wall assembly during penicillin treatment of pneumococcal mutants in which the irreversible effects of the antibiotic are blocked. In this experimental system I have discovered the production of a unique 72 kD protein, which is specifically labeled by radioactive cysteine, and which might be part of a regulatory stress-circuit mediating the global metabolic shutoff and inhibition of growth in penicillin-treated bacteria.

Chapter I

Physiological and genetic evidence for the existence of an autolysis-independent mechanism of penicillin-induced killing in pneumococci.

Summary of results

In order to clarify the relationship between penicillin-induced lysis and killing in pneumococci, we have performed a series of experiments using both autolysis-inhibiting agents as well as pneumococcal mutants defective in autolysis (Lyt^-) by virtue of a variety of alterations in the structural determinant (*lytA*) of the major autolysin (an *N*-acetylmuramyl-L-alanine amidase; referred to as amidase). We also have generated a new type of mutation (cid^-) that drastically reduced penicillin-induced killing in both wild-type (Lyt^+) and Lyt^- mutants of pneumococci. The cid^- determinant behaved like a single mutation and was genetically unlinked to the determinant (*lytA*) of the major autolysin amidase. However, in Lyt^+ cells, the cid^- mutation also reduced the "triggering" of the amidase activity (i.e., lysis) in addition to protecting the cells from penicillin-induced killing.

These results suggested the existence of a common, autolysis-independent pathway to penicillin-induced cell death in both Lyt^+ and Lyt^- pneumococci. Moreover, the fact that the cid^-

mutation reduced both penicillin-killing and lysis (while *lytA* mutations decreased principally lysis) also suggested that penicillin-treated wild-type (Lyt^+) pneumococci were first killed, and then lysed by the drug.

Materials and Methods

Strains of pneumococci and growth of the organisms. The strains of *Streptococcus pneumoniae* used in these experiments are described in table 1 (p. 25). Unless otherwise stated, the microorganisms were grown at 37°C in casein-based semisynthetic medium at pH 8 (45) supplemented with 0.1% (w/v) yeast extract (Difco Laboratories, Detroit, MI), which is referred to as C+Y. In certain experiments pneumococci were grown in the chemically defined medium Cden (A. Tomasz, Bacteriol. Proc., p 29, 1964). Growth of the cultures was monitored by their optical density at a wave length of 620 nm (OD_{620}) with a spectrophotometer (Sequoia-Turner, Mountainville, CA). Bacterial stocks were frozen in C+Y containing 10% glycerol and were stored at -70°C.

Antibiotics and reagents. Benzylpenicillin and vancomycin were obtained from Eli Lilly & Co. (Indianapolis, IN); cefoxitin and imipenem from Merck Sharp & Dohme (Rahway, NJ); cefadroxil from Beecham (Bristol, TN); and L-[4,5-^{3}H(N)]-Lysine (97.4 Ci/mmol) from Dupont NEN (Boston, MA). Antibodies against the pneumococcal autolysin amidase were prepared as described previously (15). All other chemicals were reagent grade, commercially available products.

Susceptibility testing and rate of autolysis and killing of the microorganisms. Minimal inhibitory concentrations (MIC) of

Table 1: ***S. pneumoniae*** **strains used in this study**

Phenotype and strain	*Lyt A* gene	Relevant phenotype		Origin or reference
		autolytic status	Killing by* Penicillin	
Lyt$^+$ (wild type)				
R6x	Normal	Lyt$^+$	4-5	see 57
Lyt$^-$ mutants				
Lyt 4-4	Point mutation	Lyt$^-$	3-4	"
M 31	Deletion	Lyt$^-$	3-4	"
RUP-24	Plasmid insert. inactivation	Lyt$^-$	3-4	"
Cid$^-$ mutant				
Cid-1	Normal	Lyt$^+$	1	this work
Lyt$^-$ Cid$^-$ recombinants				
Lyt 4-4 x Cid-1 (= strain T6)	Point mutation	Lyt$^-$	1	this work
RUP-24 x Cid-1	Plasmid insert. inactivation	Lyt$^-$	1	this work
Lyt$^+$ Cid$^-$ recombinants				
Lyt$^-$ Cid$^-$ x R6x	Normal	Lyt$^+$	1-2	this work

*Loss of log 10 CFU after 6 h of treatment with a 20x MIC of Penicillin
Note that the MIC values of the strains in the Table were similar, i.e., 0.01 μg/ml

antibiotics for the organisms were determined by the tube dilution method in C+Y, using 1-ml volumes per tube and 10^5 CFU/ml as inocula. The MIC was defined as the lowest antibiotic concentration inhibiting visible bacterial growth after 24 h of incubation at 37°C. Rates of autolysis were measured in 10-ml cultures of exponentially growing *S. pneumoniae* (OD_{620} = 0.2 to 0.3, corresponding to 0.5 to 1 x 10^8 CFU/ml) that received various concentrations of the antibiotics (ranging from 10x to 100x the MIC). Control cultures, which were used for the evaluation of stationary-phase lysis, received no antibiotics. Autolysis rates were expressed as the first-order rate constant K, where $K = \ln(A_0/A_{120}) \times \min^{-1}$, and where A_0 represents the peak OD_{620} reading (usually observed shortly after addition of the drug, see Results section) and A_{120} represents the reading after a further 120 min of incubation (49). To determine the effect of antibiotic treatment on viable counts of bacteria, 100-μl portions of the cultures were removed after various times of exposure, serially diluted in semisynthetic medium supplemented with 1000 U of penicillinase/ml (Becton Dickinson, Cockeysville, MD), and plated onto tryptic soy agar plates (Difco) containing 3% of defibrinated sheep's blood. The CFU were counted after 36 h of incubation at 37°C.

Inhibition of pneumococcal autolysis by specific inhibitors of amidase. Cultures of the lysis-prone (Lyt^+) wild-type strain R6x (see table 1, p. 25) were grown to the early exponential phase

(about 0.5 x 10^8 CFU/ml) and treated with the following autolysin-inhibitory agents: (i) a high concentration of choline (10 mg/ml) (7,26), (ii) antiserum prepared against the autolysin amidase (15) (100 μl of serum per ml) and (iii), trypsin (50) (100 μg/ml). Incubation continued for an additional mass doubling time, when the cultures received 0.1 μg of penicillin/ml (i.e., 10x the MIC). The OD_{620} and viable titers were then followed as described above.

Selection of pneumococcal mutants defective in penicillin-induced killing and lysis. A nonmutagenized culture of R6x (in 10 ml of C+Y; see table 1, p. 25) in the exponential phase of growth (1 x 10^8 CFU/ml) was treated for 4-6 h with 20 x MIC of benzylpenicillin. The cells were then washed three times by centrifugation (10,000 g for 10 min) and resuspended in penicillin-free medium, and the survivors were grown overnight in fresh C+Y. Such passages were repeated several times and are referred to as "enrichment cycles". After 18 passages the cells were again exposed to 20 x MIC of penicillin (cycle 19) for 6 h and plated for colony count. Nine surviving colonies (all originating from the same initial culture) were picked at random from the plates, re-exposed to penicillin (cycle 20), and designated Cid-1 to Cid-9.

Autolysin specific activity. The autolytic activity in crude extracts of pneumococci was measured by using a published method

(33) with slight modifications. In short, exponential-phase pneumococci were suspended in 500 µl of ice-cold TMB (0.05 M Tris maleate buffer at pH 7.0, plus 0.1% of polyoxy-ethylene lauryl ether) and lysed by sonication for 10 min. The lysates were centrifuged (12,000 g for 10 min) and the supernatants serially diluted in TMB. Samples (25 µl) of each dilution were added to 210 µl of TMB containing 15 ug of cell walls biosynthetically labeled with radioactive L-[4,5-^{3}H(N)]-Lysine (3000 cpm/ug of cell wall) and incubated at 37°C for 30 min. The samples were then rapidly chilled, and the non-degraded cell wall material was removed by centrifugation (12,000 g for 10 min). Radioactivity in the supernatant solution was determined by pipetting 50 µl-portions into 5 ml of Ready-Solv scintillator (Beckman, Palo Alto, CA) and counting the samples in a Mark II scintillation spectrometer (Nuclear Chicago, Chicago, IL). Protein concentrations were determined by the bicinchoninic acid protein assay (described by Smith et al. (75A) and available in commercially obtainable kits: Pierce Chemical, Rockford, IL).

Genetic transformation. Chromosomal DNA was prepared by the method of Marmur (52). Competent pneumococci were prepared by a previously published method (78). Competent cells (about 0.5 x 10^{8} CFU) were incubated with 0.1 to 1 ug of transforming DNA/ml at 30°C for 30 min, followed by the addition of DNase (final concentration, 0.1 µg/ml) and an expression time of 90 min at 37°C. Transformants were either screened for their lytic response

to deoxycholate (DOC) using a membrane assay (Millipore Corp., Bedford, MA) described previously (16), or selected for their ability to resist killing by penicillin (Cid^- transformants, see below) or for resistance to streptomycin.

Transformation of the *cid*$^-$ determinant. High-molecular weight DNA prepared from a pneumococcal mutant (Cid-1; see table 1, p. 25) that was highly resistant to the killing effect of penicillin (Cid^-phenotype) was used to transfer the kill resistance marker (the *cid*$^-$ determinant) into kill-sensitive (Cid^+ phenotype) competent cells with defective or inactivated *lytA* genes (strains Lyt 4-4 and RUP-24, Lyt^- phenotype; see table 1). As a control, competent cells were treated with DNA from the lysis-prone and highly kill-sensitive wild-type strain R6x carrying a streptomycin resistance (Str) marker (strain R6xStr). Cid^- transformants were selected by two methods, first in liquid medium and second on agar plates.

(i) Selection in liquid medium. Pneumococcal cultures that were treated with either Cid-1 or R6xStr DNA were exposed to consecutive enrichment cycles of treatment with 20x the MIC of penicillin for 6 h, followed by outgrowth of the survivors in drug-free medium. Such consecutive enrichment cycles select for Cid^- cells (see Results section).

(ii) Selection on agar plates. Selection on agar plates was aimed at the determination of the frequency of transformation of the *cid*$^-$ determinant. Ten thousand CFU from cultures treated with either Cid-1 or R6xStr DNA were plated onto C+Y agar plates (12 ml of 1.5% C+Y agar plus 250 U of catalase/ml) and were subsequently overlaid with 8 ml of the same medium and incubated for 5 h at 37°C, in order to allow each cell to form a micro-colony (or miniculture). Then, a solution of penicillin (1 ml of a 10 μg/ml preparation) was overlaid and allowed to seep into the agar, and the incubation was continued for 12 h, after which time penicillinase (1ml of a 10^5-U/ml solution) was spread onto the agar plates, in order to inactivate the antibiotic. Bacteria that survived penicillin treatment were counted after an additional 48 h of incubation at 37°C.

Statistical analysis. Differences between means were calculated by the Student *t* test.

Results

Effect of autolysis-inhibiting agents on penicillin-induced killing of pneumococci. Figure 3 (p. 32) shows that while inhibitory concentrations of each of the autolysis-inhibiting agents (i.e., choline, trypsin, or anti-amidase serum) prevented culture lysis (as measured by the decline in OD_{620}), there was virtually no protection from the loss of viability in the presence of lysis-inhibiting concentrations of choline. Similar results were also obtained when trypsin or anti-amidase serum were used in place of choline.

Rate of penicillin-induced killing in pneumococcal mutants defective in amidase activity. Cultures of Lyt^- mutants Lyt 4-4, M31 and RUP-24 (table 1, p. 25) in the exponential phase of growth received penicillin (10x, 20x, or 100x the MIC), and the effect of the drug on the OD_{620} and the viable titer of the cultures was followed. None of the mutant cultures showed significant penicillin-induced lysis, yet substantial loss of viability continued in all three mutants, albeit at a slower rate than that of the wild-type (Lyt^+) cells. Figure 4 (p. 34) shows the number of surviving CFU in cultures of isogeneic Lyt^+ (R6x) and Lyt^- (RUP-24) strains (table 1) that received 100x the MIC of penicillin at a cell concentration of 10^8 CFU/ml (corresponding to an OD_{620} of 0.25) and were followed for 6 h. The number of survivors in RUP-24 (Lyt^-) cultures was $2.8 \times 10^4 \pm 1.3 \times 10^4$

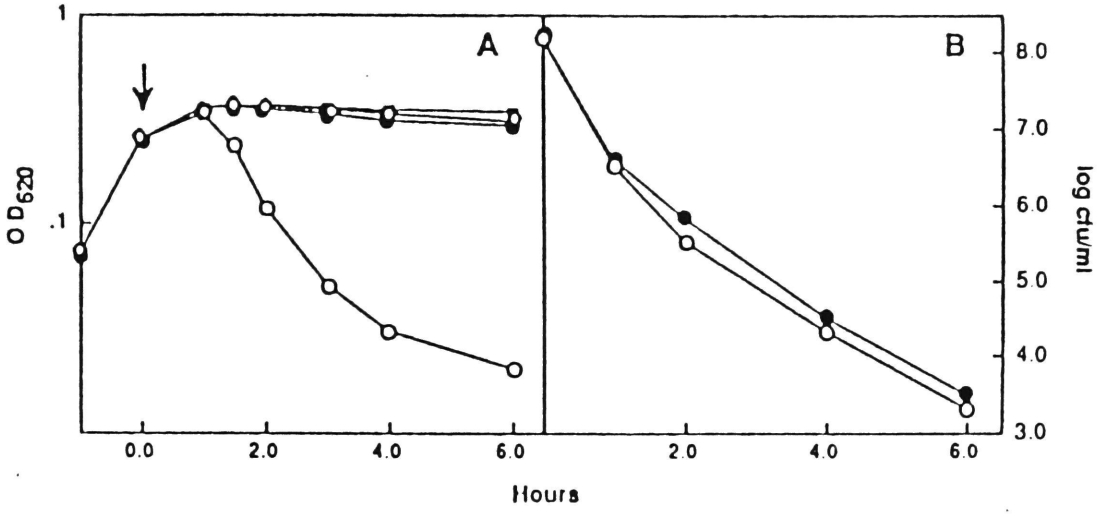


Figure 3: **Effect of various specific inhibitors of the autolytic enzyme amidase on penicillin-induced lysis (A) and loss of viability (B) of the lysis-prone (Lyt^+) and highly kill-sensitive wild-type strain R6x. Cultures (10ml) of R6x in the early exponential phase of growth (about 0.5x 10^8 CFU/ml) received high concentrations of choline (closed circles), amidase antiserum (closed squares) or trypsin (open diamonds) and were incubated for an additional doubling time, when they were treated with 10x the MIC of penicillin (arrow). Control cultures (open circles) received only penicillin treatment. The OD_{620} of the cultures and bacterial survival were followed for 6 h.**

(mean ± standard deviation of 19 determinations in three separate experiments). In comparison, in R6x (Lyt^+) cultures the number of survivors was $2.1 \times 10^3 \pm 1 \times 10^3$ (mean ± standard deviation of 17 determinations in three separate experiments), which was significantly lower than the numbers of survivors in Lyt^- cells ($P < 0.001$). Similar results were obtained after exposure of the same isogeneic pair of pneumococci to 10x or 20x the MIC of penicillin or when the Lyt^- mutants Lyt 4-4 and M31 were used in place of RUP-24 (see strains in table 1, p. 25). Thus, suppression of the major autolysin amidase by either structural mutations or deletion of the *lytA* gene resulted in a statistically significant reduction of penicillin-induced killing of pneumococci (3 to 4 Log units of killing per 6 h compared with 4 to 5 Log units of killing per 6 h in the parental Lyt^+ strain; $P < 0.001$). However, loss of viability continued at a considerable rate in Lyt^- mutants (3-4 Log units per 6 h) whether the lytic defect was due to either a point mutation, insertion-inactivation, or a complete deletion of the *lytA* gene. This finding shows that the residual killing of Lyt^- pneumococci by penicillin does not involve the autolytic amidase, but some other as yet undefined mechanism(s).

In order to study such autolysis-independent mechanism(s) of killing, we have generated pneumococcal mutants with greatly decreased sensitivity to penicillin-induced killing. These experiments are described next.

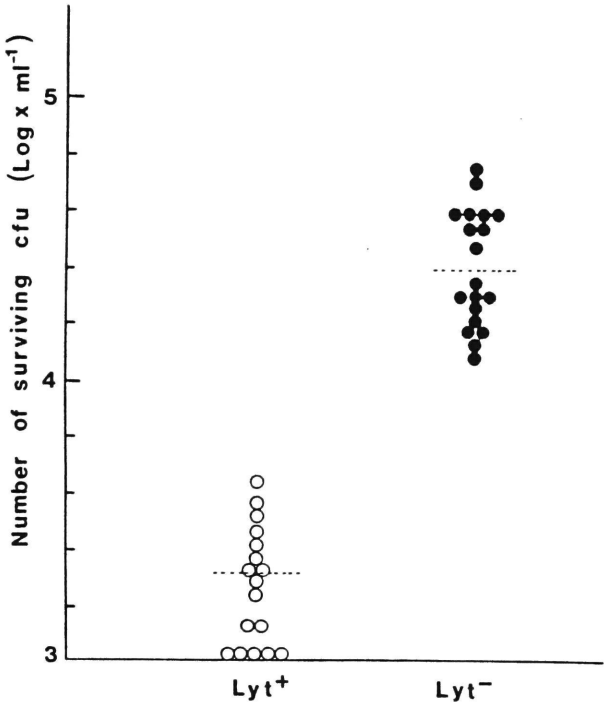


Figure 4: Number of surviving CFU of the Lyt$^+$ wild-type strain R6x (open circles) and the Lyt$^-$ strain RUP-24 containing an insertionally inactivated amidase (*lytA*) gene (closed circles) after 6 h of treatment with 100x the MIC of penicillin. Cultures (10 ml) of strains in the exponential phase of growth (10^8 CFU/ml) were treated with penicillin for 6 h, and the numbers of surviving CFU were determined. Horizontal dashed lines represent the mean values of the number of survivors. Mean $\pm$ standard deviations of the surviving CFU of strain R6x and RUP-24 were statistically different ($P < 0.001$).

Selection of pneumococcal mutants with decreased sensitivity to penicillin-induced killing (Cid$^-$) and lysis. Mutants with greatly decreased susceptibility to penicillin-induced killing (referred to as Cid$^-$ phenotype, for bactericidal) were generated by cyclic exposure of a penicillin-susceptible, lysis-prone (Lyt$^+$) parental strain of pneumococcus (strain R6x; table 1, p. 25) to 20x the MIC of penicillin for 4-6 h, followed by removal of the drug and outgrowth of the culture in antibiotic-free medium (see Materials and Methods). Such enrichment techniques mimicked the situation in the clinical setting, where bacteria may be exposed cyclically to transient high (supra-MIC) concentrations of penicillin followed by prolonged lower (sub-MIC) levels of the drug. Colonies surviving the 20th cycle of penicillin treatment showed a greatly decreased susceptibility to both penicillin-induced killing and lysis.

Nine independent colonies (Cid-1 to Cid-9, originating from the same initial culture) isolated in this manner had unchanged MIC values of penicillin (0.01 μg/ml) and undiminished sensitivity to lysis by DOC. On the other hand, each of the nine mutants showed greatly decreased sensitivity to both penicillin-induced killing (loss of viability of 1-2 Log per 4-6 h in the mutant culture vs. 4-5 Log for the parent strain R6x) and lysis (K x 10^3 values, 0.3-1.7 in the mutant cultures vs. 22.0 for the parent cells; see table 2, p. 37), at both 37°C or 32°C.

Importantly, selection of kill-resistant mutants by cyclic exposure to penicillin was not a property restricted to the

laboratory pneumococcal strain R6x. Cycling of the *lytA* deletion or insertion-inactivated mutants M31 and RUP-24 (table 1, p. 25) or of a penicillin-resistant clinical isolate of pneumococcus (strain D23, MIC of penicillin 0.12, capsular type 2) also generated mutants able to survive treatment with high concentrations of penicillin (data not presented).

Characterization of the kill-resistant and lysis-defective mutant Cid-1. A single mutant - Cid-1 - was chosen for detailed studies. The mutant Cid-1 originated from a single (10 ml) non-mutagenized culture of the parent strain R6x, which received the first penicillin treatment at a cell concentration of 10^8 CFU/ml, a result suggesting that the spontaneous frequency for this mutation was probably greater than 10^{-9} (this assumption was not based on experimental result). The kill-resistance and defective penicillin-induced lysis characterized each of the nine randomly picked mutants Cid-1 to Cid-9 (see above). However, serial passage of the mutants in drug-free medium or repeated freezing and thawing resulted in gradual loss (reversion) of both phenotypes. This appeared to be due to the overgrowth of the mutant cultures by revertant cells, which were detectable at a frequency of about 10^{-4} to 10^{-5} in growing cultures and had a slightly shorter lag phase (compared to the mutant cells) when regrown from frozen stock into liquid cultures (see Appendix I).

The lysis defect of Cid-1 was not specific for benzylpenicillin, but was shared by a number of other beta-lactam

Table 2: **Rates of autolysis and killing of R6x (parent) and Cid-1 (Cid$^-$) when exposed to 20 x MIC of various β-lactam antibiotics and vancomycin.**

	R6x			Cid-1		
Antibiotic	MIC(μg / ml)	Lysis (K x 10^3)*	% survival §	MIC (μg / ml)	Lysis (K x 10^3)*	% survival §
Penicillin	0.01	22	0.05	0.01	0.5	20
Cephalothin	0.075	11	0.1	0.15	0.5	10
Cefoxitin	1.5	30	0.003	3.0	0.8	8
Cefadroxil	3.0	18	0.27	3.0	0.6	10
Cephalexin	4.7	18	0.26	4.7	0.6	13
Imipenem	0.015	35	0.03	0.015	4.0	0.8
CGP 31608	2.5	23	0.006	2.5	2.0	1.2
Vancomycin	0.1	22	0.01	0.1	20.0	0.1

* $K = [\ln(A_0 / A_{120})]$ min., where A_0 represents the peak absorbance reading at OD 620 and A_{120} the reading after an additional 120 min of incubation.

§ Percentage of cells surviving after 4 h of antibiotic treatment.

antibiotics tested (table 2, p. 37) and also by D-cycloserine, an antibiotic which inhibits an earlier step of cell wall synthesis (i.e., the ligation of D-Ala to D-ala which provides the ubiquitous D-Ala-D-Ala terminus to the precursors of the cell wall peptidoglycan). Interestingly, two beta-lactam antibiotics of novel structure,imipenem and CGP 31608 (an investigational penem antibiotic; CIBA-GEIGY, Basel, Switzerland), and the non beta-lactam cell-wall inhibitor vancomycin could still lyse and kill Cid-1, although the rates were slower (ca. 10x) than those of the parent strain.

Cid-1 contained an autolytic *N*-acetylmuramyl-L-alanine amidase of specific activity equal to that of the parent R6x, as indicated by identical capacities of crude extracts of both strains to depolymerize >90% of pneumococcal cell walls labeled with radioactive lysine (documented in tables 3 and 4, p. 40 and 43 resp.). The normal lysis and >90% cell degradation of Cid-1 treated with DOC (and to some extent with some penem antibiotics and vancomycin, see table 2) indicated that the *cid*$^-$ mutation did not cause gross alteration in the structure of the cell wall (see Chapter III for details).

Genetic characterization of the mutant Cid-1 (Cid$^-$ phenotype). The ability of the Cid$^-$ mutants to survive penicillin was first thought to be the consequence of defective lysis during antibiotic treatment. However, the continued penicillin-induced killing of Lyt$^-$ mutants completely lacking amidase activity

clearly excludes this explanation. The kill resistance observed in the Cid-1 mutant (illustrated in figure 5, p. 46) must be the result of some alteration in a second, as yet undefined, bactericidal target. To better define this second target, we attempted to characterize the mutation conferring the Cid$^-$ phenotype (provisionally called *cid*$^-$) at the genetic level. These experiments are described next.

(i) The *lytA* gene and the *cid*$^-$ mutation are unlinked genetic determinants. Since the Cid-1 mutant was resistant to penicillin-induced lysis, it was important to determine whether the *cid*$^-$ was not merely a new *lytA* allele. To test this possibility, chromosomal DNA of strain Cid-1 (Cid$^-$ Lyt$^+$) was used to transform RUP-24 (Cid$^+$ Lyt$^-$) recipient cells to the Lyt$^+$ phenotype. Transformants were screened and chosen on the basis of lysis when exposed to DOC. Lyt$^+$ transformants were tested for penicillin-induced killing and lysis. Note that RUP-24 is erythromycin-resistant (Erm) due to insertion-inactivation of its *lytA* gene with an *em* determinant (*lytA::em* genotype; see tables 1 and 3, p. 25 and 40 resp.). Therefore Lyt$^+$ transformants of RUP-24 were also expected to be erythromycin-sensitive (ErmS) due to the conversion of their *lytA::em* allele by the *lytA* gene of Cid-1.

The results are summarized in table 3 (p. 40). As expected, all 116 independent Lyt$^+$ transformants had lost their Erm marker along with the transformation to the Lyt$^+$ phenotype. When

Table 3: Cid-1 contains a "normal" *lyt A* gene that does not cotransform with the *cid*⁻ determinant in genetic crosses .

Status in genetic cross	Bacterial isolate	Erm *	lysis		Penicillin⁺ killing	Amidase activity	Assigned Phenotype
			DOC	Penicillin			
DNA donor	Cid-1	S	+	_	1	100%	Cid⁻ Lyt⁺
Recipient	RUP-24	R	_	_	3-4	0.1-1%	Cid⁺ Lyt⁻
Transformants§	116 indep. clones	S	+	+	4-5	100%⁺⁺	Cid⁺ Lyt⁺
W⁻T control	R6x	S	+	+	4-5	100%	Cid⁺ Lyt⁺

* Erythromycin resistant

\+ Cells were treated with 20 x MIC of penicillin for 6h. Survival is expressed as the loss of Log units CFU/ml during the treatment.

⁺⁺ Amidase activity was measured in one representative of the 116 Lyt⁺ transformants .

§ All the 116 independant Lyt⁺ transformants behaved identically in the various tests described in the Table .

challenged with 20x the MIC of penicillin, all transformants were also rapidly killed and lysed by the antibiotic, like the wild-type strain R6x (Cid^+, Lyt^+; see table 3). These results indicated (i) that the mutant Cid-1 carried a "normal" *lytA* determinant that was able to function like a wild-type gene when transformed back into a Cid^+ background, and (ii) that the *cid*$^-$ determinant was not carried along with the *lytA* gene in DNA transformation. Thus, the determinants were genetically unlinked.

This latter observation was further confirmed in the reverse cross, shown in the next set of experiments.

(ii) Transformation of the *cid*$^-$ determinant into pneumococcal recipients lacking the autolysin amidase (Lyt^-). Since both the *cid*$^-$ and the *lytA* determinants were genetically unlinked, the question arose as to whether *cid*$^-$ might also confer the Cid^- phenotype to recipient pneumococci lacking the autolysin amidase. To answer this question, we attempted to transform kill-sensitive pneumococcal *lytA* mutants (Cid^+, Lyt^-) to the Cid^- phenotype, using DNA of the Cid^- Lyt^+ strain Cid-1. Two methods of selection were used, one in liquid medium and one on agar plates.

(a) Selection in liquid medium. Cultures of Lyt^- pneumococci sensitive to the killing action of penicillin (i.e., Cid^+, Lyt^- strains) were treated with transforming DNA prepared from the kill-resistant mutant Cid-1. Such cultures were quantitatively converted to the Cid^- phenotype (1 Log unit

killing per 6 h of penicillin treatment) after three to four enrichment cycles, whereas those treated with the control R6xStr DNA (rate of transformation to Str 0.1-1%) were still rapidly killed after six to nine cycles of penicillin treatment (3-4 Log units of killing per 6 h). Table 4 (p. 43) shows the properties of five such Cid$^-$ transformants that were randomly picked from the plates of two crosses in which either Lyt 4-4 or RUP-24 was used as the recipient. All the transformants showed the reduced bactericidal sensitivity of the DNA donor, while they retained the Lyt$^-$ phenotype of the recipients.

(b) Selection on agar plates. Selection on agar plates allowed an approximate estimation of the frequency of transformation of the *cid*$^-$ determinant. In a typical experiment, 11 of 10^4 (0.1%) CFU treated with Cid-1 DNA survived the penicillin treatment. Upon testing the bactericidal sensitivity of these colonies in liquid cultures, 10 of them showed the typical Cid$^-$ phenotype, but none had converted to the Lyt$^+$ phenotype. No survivors were detected among the 10^4 bacteria treated with R6xStr DNA (transformation rate to Str of 0.1-1%). These observations suggested a frequency of transformation of about 0.1% for the *cid*$^-$ determinant, which was consistent with that of a single mutation.

Thus, the *cid*$^-$ determinant could confer resistance to penicillin-induced killing in either Lyt$^+$ or Lyt$^-$ pneumococci and did not cotransform with the *lytA* gene. This confirmed our

Table 4: The *cid*⁻ determinant also confers "kill-resistance" to Lyt⁻ pneumococci.

Status in genetic cross	Bacterial isolate	Erm *	lysis		Penicillin⁺ killing	Amidase activity	Phenotype assigned
			DOC	Penicillin			
DNA donor	Cid-1	S	+	_	1	100 %	Cid⁻ Lyt⁺
Recipient	RUP-24 or Lyt 4 -4	R	_	_	3-4	0.1-1 %	Cid⁺ Lyt⁻
Transformants	5 indep. transf. of each recipient	R	_	_	1	0.1-1 %	Cid⁻ Lyt⁻

*Erm is applicable only when strain RUP-24 is used as a recipient (strain Lyt 4-4 is Erm susceptible).
⁺Cells were treated with 20x the MIC of penicillin for 6 h. Survival is expressed as the loss of Log units CFU/ml during the treatment.

previous observation that both determinants were genetically unlinked (see above), and also raised the possibility that *cid*$^-$ and *lytA* might be functionally independent.

(iii) Introduction of the lytA gene in Cid$^-$ Lyt$^-$ transformants. In order to test whether one marker could affect the physiological expression of the other, a "wild-type" *lytA* gene was reintroduced in a Cid$^-$ Lyt$^-$ mutant (strain T6) constructed in the present experiment, and the response of the Lyt$^+$ transformants to penicillin treatment was analyzed.

Table 5 (p. 45) shows the properties of six such Lyt$^+$ transformants. All of them were lysed by DOC and contained normal (wild-type) levels of amidase activity. However, both their rates of lysis and killing by penicillin were intermediate between the values typical of the DNA recipient and the DNA donor strains (Figure 5, p. 46).

Thus, while the *cid*$^-$ and *lytA* determinants are genetically distinct, there is a functional relationship between both determinants during penicillin treatment. The *cid*$^-$ mutation is able to control the rate of penicillin-induced lysis in Lyt$^+$ cells whereas the presence of *lytA* influences (although marginally) the rate of killing in Cid$^-$ bacteria.

Table 5: The *cid*$^-$ determinant controls both penicillin-induced killing and lysis in Lyt$^+$ cells.

Status in genetic cross	Bacterial isolate	lysis		Penicillin$^+$ killing	Amidase activity	Phenotype assigned
		DOC	Penicillin			
DNA donor	R6x	+	+	4-5	100 %	Cid$^+$ Lyt$^+$
Recipient	T6	–	–	1	0.1-1 %	Cid$^-$ Lyt$^-$
Transformants	6 indep. transf. tested	+	–	1-2	100 %	Cid$^-$ Lyt$^+$

$^+$ Cells were treated with 20x the MIC of penicillin. Survival is expressed as the loss of Log units CFU/ml during the treatment.

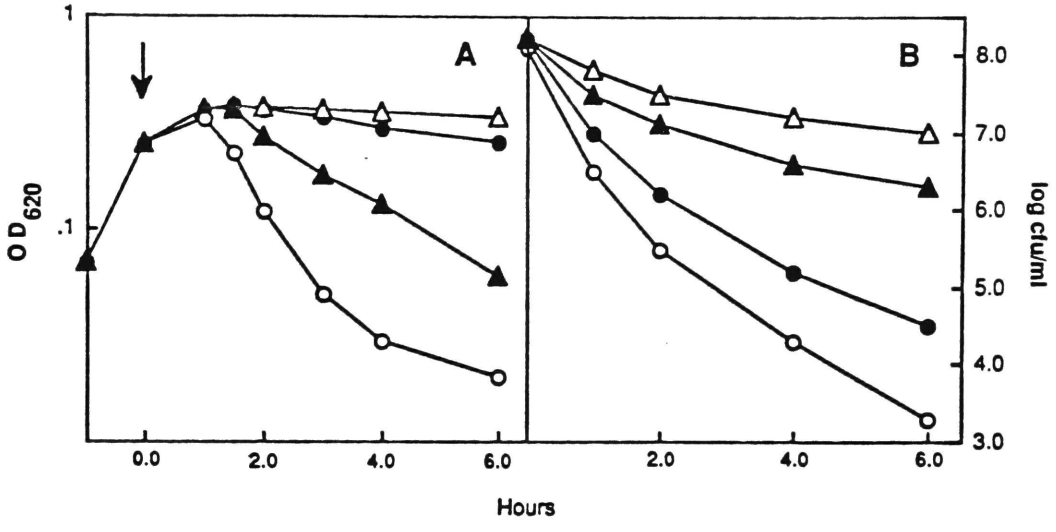


Figure 5: Penicillin-induced lysis (A) and loss of viability (B) of the Lyt^+ wild-type strain R6x (open circles), the Lyt^- strain RUP-24 containing an insertionally inactivated amidase gene (closed circles), the kill-resistant mutant Cid-1 (open triangle) and the $Cid^- Lyt^+$ recombinant (closed triangles). Cultures (10 ml) of the strains in exponential phase of growth (10^8 CFU/ml) were treated with 20x the MIC of penicillin (arrow), and both the OD_{620} and bacterial survival were followed for 6 h.

Discussion

The experimental results described here demonstrate the existence of two distinct targets or mechanisms for the killing effect of penicillin (as well as other cell wall inhibitors) in pneumococci. These two (autolysis-independent and autolysis-dependent) targets could be identified by careful quantitation of the susceptibilities of wild-type pneumococci (Cid^+ Lyt^+) and two kinds of autolysin defective pneumococcal mutants (Cid^+ Lyt^- and Cid^- Lyt^- cells), to the bactericidal effect of penicillin. Under the conditions of our assays (exposure to 20x the MIC of penicillin for 4-6 h), each one of these strains lost viable titers at characteristic, highly reproducible rates. Wild-type Cid^+ Lyt^+ cells lost 4-5 Log units of viable counts per 6 h, Cid^+ Lyt^- mutants lost 3-4 Log units viable counts per 6 h (a protection of 1 Log unit that resulted from the inactivation of the *lytA* gene), and Cid^- Lyt^- cells lost 1 Log unit of viable count per 6 h. Reintroduction of the *lytA* gene into either Cid^+ Lyt^- of Cid^- Lyt^- strains caused an increase in the rate of killing by approximately 1 Log unit per 6 h. These data indicate that in the wild-type strains, triggering of the amidase activity is responsible for about 1 Log unit of viability loss, while the rest of the killing (3-4 Log units) occurs by a second, amidase independent mechanism.

This second bactericidal target has been identified in genetic terms. The cid^- marker is distinct from the genetic

determinant of the autolysin amidase (*lytA*) and it shows no genetic linkage to *lytA* in DNA transformation. Moreover, the protective effect of the *cid*$^-$ mutation is fully expressed in pneumococci completely lacking the amidase.

At this point, it is useful to remember that the autolysin amidase is dispensable for normal growth of pneumococci both *in vitro* and *in vivo* (in a mouse model of infection) (6,81). Therefore, if amidase-induced lysis was to be the primary effector in penicillin-induced killing, one would expect mutants to arise either in the *lytA* gene itself, or in its substrate, the bacterial cell wall. Instead, however, the *cid*$^-$ mutation appeared to control another target of penicillin-induced killing which was operative in both amidase-free (Lyt$^-$) and wild-type (Lyt$^+$) pneumococci. These observations suggest the existence of a common pathway to cell death during penicillin-induced killing in both Lyt$^+$ and Lyt$^-$ pneumococci. This common pathway is controlled by *cid*$^-$, because the mutation can protect both types of cells from penicillin-induced killing, but not *lytA*, because inactivation of this gene only marginally affects penicillin-induced killing. This suggests that during penicillin treatment, wild-type (Lyt$^+$) pneumococci are first killed and then lysed by the drug (see figure 6, p. 50).

While this model determines an order for the physiological interplay of the *cid*$^-$ and *lytA* determinants during penicillin-induced killing and lysis of pneumococci, it does not specify the nature or the number of events involved in the process.

Experiments aimed at defining the nature of the "killing lesion" inflicted by penicillin to the cells are presented in Chapter III of the present thesis dissertation.

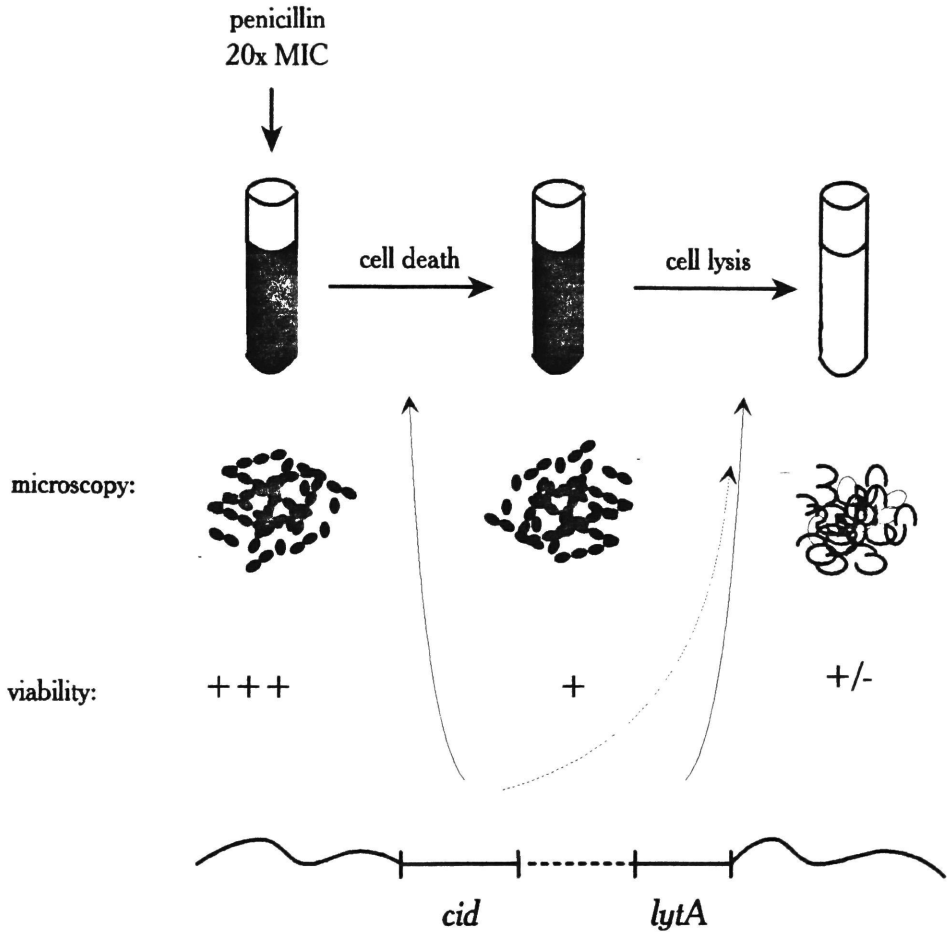


Figure 6: Model for the interplay of the *cid* and *lytA* genetic determinants in penicillin-induced killing of pneumococci. The bacteria are first killed and then lysed. The *cid* gene (or locus) controls both penicillin-induced killing (left plain arrow in the figure) and, to a lesser extend, lysis by amidase (dotted arrow). The *lytA* gene, in contrast, controls essentially lysis (right plain arrow), but influences only marginally penicillin-induced killing (see text for details).

Chapter II

Molecular characterization of the *cid*$^-$ marker.

Summary of results

This chapter describes several approaches tried to characterize the *cid*$^-$ mutation at the molecular level. First we attempted to generate Cid$^-$ mutants by shotgun mutagenesis of Cid$^+$ cells, using either one of the following techniques: (i) insertion-duplication mutagenesis with a so-called suicide vector carrying an antibiotic-resistance marker, and (ii) transposon mutagenesis, using the conjugative transposons Tn*1545* or Tn*916* (11,73). Second, we attempted to clone the *cid*$^-$ determinant by a technique of gene conversion, using *E. coli* as an intermediate host.

While both techniques are complementary approaches to gene cloning, they did not allow the molecular isolation of the *cid*$^-$ determinant. This suggested the possibility that the nature of the *cid* gene was both (i) incompatible with survival of null-mutants (because insertion-inactivated Cid$^-$ mutants could not be constructed) and (ii), potentially toxic for the *E. coli* intermediate host (because the *cid*$^-$ determinant appeared to be lost during passage through *E. coli*).

Materials and Methods.

Bacterial strains and growth conditions. Five bacterial strains were used in addition to those used in Chapter I. Strain *E. feacalis* BM4110 (Str/Tn*1545* [Km, Erm, Tc]; kindly provided by Dr. Poyart-Salmeron, Institut Pasteur, Paris, France) was used as a donor in conjugative transfer of Tn*1545* to pneumococci (11). Strain *E. feacalis* CG110 (Fus, Rif/Tn*916* [Tc]; provided by Dr. Masure from the Rockefeller University, New york) was used in conjugative transfer of Tn*916* (73). Two derivatives of the Lyt$^-$ mutant Lyt 4-4 were used as recipients in conjugation experiments, one carrying a Nov marker (to select against the Tn*1545* donor strain) and one carrying an Str marker (to select against the Tn*916* donor). Finally, *E. coli* DH5α [in (1)] was used as an intermediate host for cloning pneumococcal DNA. Unless otherwise stated, *E. feacalis* were grown in brain heart infusion (BHI, Difco) or agar, and *E. coli* in Luria-Bertani (LB) medium [in (1)].

Antibiotics and reagents. Erythromycin, tetracycline and novobiocin were purchased from Sigma Chemical Company (St. Louis, MO), the restriction enzymes *Bcl*1, *BamH*1, *Cla*1 and *Taq*1 from New England Biolabs (Beverly,MA) and *EcoR*1, *Sau3*A and T*4* ligase from BRL (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD). All other chemicals were reagent grade commercially available products.

Plasmid and DNA preparation. The plasmid pVA894 (kindly provided by Dr. Pozzi, University of Sienna, Sienna, Italy; (90)), pJCD9 (9) and pGL80 (14) were used in these experiments. Both pVA894 and pJCD9 are *E. coli* vectors that cannot replicate autonomously in pneumococci. However, both vectors contain an Erm marker which can be expressed in the latter organism, provided that the whole plasmid is inserted in the pneumococcal chromosome (89). These vectors were used in insertion-duplication mutagenesis of pneumococci (see Results section). The plasmid pGL80 is an *E. coli* vector that contains an Amp marker as well as the cloned pneumococcal *lytA* gene (14).

Large scale plasmid preparations were purified by differential centrifugation in CsCl and "mini-preparations" by the rapid alkaline-lysis method [in (1)]. Pneumococcal chromosomal DNA was prepared by the Marmur method (52). Restriction enzymes and T4 ligase were used according to the manufacturers instructions. Ligation of pneumococcal target DNA fragments in plasmid vectors was performed at molar concentration ratios of 1/10 (target/vector) without dephosphorylation.

In certain experiments, DNA fragments of specific size ranges were isolated from chromosomal digests. This was achieved either by recovery of the fragments after separation by electrophoresis in low melting point agarose or by fractionation of partial DNA digests on sucrose density gradients (see next).

For partial digestion, 1000 μg of chromosomal DNA from the Cid^- mutant Cid-1 (table 1, p. 25) in TE (20 mM Tris HCl pH8 and

5 mM of EDTA) plus adequate amounts of restriction enzyme buffer were distributed in 9 Eppendorf tubes (450 µl in tube #1 and 250 µl in the remaining tubes). Fifty µl of a solution containing 250 U of the appropriate restriction enzyme were added in tube #1 and 2x-serially diluted over the other tubes, except for tube #9, which received no enzyme. Digestion was allowed for 1 h at 37°C and stopped by heat inactivation of the enzyme for 20 min at 57°C. The quality of the digest was controlled by agarose gel electrophoresis.

For size separation by sucrose gradient density, the DNA of the 9 tubes was pooled and overlaid on a 10-40% sucrose gradient (in 35 ml of 1 M NaCl, 20 mM Tris HCl pH8 and 5 mM of EDTA) contained in SW28 tubes. After centrifugation for 20 h at 24,000 rpm (at 20°C) fractions of about 1 ml were collected. The fractions were tested both for appropriate size separation by agarose gel electrophoresis and for their ability to transform the Cid^- phenotype to Cid^+ pneumococci (using penicillin cycling, see Chapter I). DNA fragments from suitable fractions were dialyzed in TE and concentrated by ethanol precipitation before being used for ligation in *E. coli* vectors.

Conjugation and DNA transformation. Conjugative transfer of Tn*1545* and Tn*916* was performed by slight modifications of the filter mating method (11,73). Liquid cultures of both donor and recipient strains were grown to late logarithmic phase (OD_{620} of 0.6-0.7), mixed at a ratio of 1/10 (1 ml of the donor culture for

9 ml of the recipient culture) centrifuged, suspended in 200 µl of fresh BHI broth and spread on Millipore filters (0.45 µg) on top of BHI agar plates supplemented with 3% of defibrinated sheep blood. The diameters of the filters were 25 mm and 45 mm in experiments with Tn*1545* and Tn*916* respectively. After 16 to 20 h of incubation at 37°C, pneumococcal transconjugants were selected by either of the following techniques. (i) For Tn*1545*, the bacteria were washed from the filters with 2 ml of medium, and plated on BHI agar supplemented with 10 ug of novobiocin/ml (to select against the donor strains) and 1 ug of erythromycin/ml (to select for the transposon). (ii) For Tn*916*, the filters were replica plated on blood agar plates containing 100 ug of streptomycin/ml (to select against the donor) and 10 ug of tetracycline/ml (to select for the transposon). This latter technique permitted to isolate the progeny of independent pneumococcal transconjugants.

DNA transformation in pneumococci was as described in Chapter I. Inducible erythromycin resistance was selected using a "sandwich" plating technique. Petri dishes containing a first 8 ml agar layer were overlaid with a second 8 ml layer of blood agar containing adequate dilutions of the transformed bacteria. Expression of transformed genes was allowed for 90 min at 37°C, after which a new 8 ml of agar (buffer layer) was poured, allowed to solidify and overlaid by a last 8 ml of agar containing 3 ug of erythromycin/ml. The plates were then incubated for 24-48 h at 37°C before erythromycin resistant transformants were counted.

To transform *E. coli* DH5α, 50 µl of an overnight culture in LB broth were inoculated in 5 ml of fresh medium, grown for 3 h at 30°C in a shaking incubator, chilled on ice, pelleted and suspended in 75 µl of fresh prechilled LB broth. After 5 min on ice, the cells were gently mixed with and additional 75 µl of so-called 2x-TSS (see below) and left 5 more min on ice before DNA was added. Incubation with DNA on ice was allowed for another 30 to 60 min, before a 2 min heat shock (42°C) was applied followed by rapid chilling and addition of 1 ml of fresh LB broth to the tubes. A 90 min expression time at 37°C in a shaking incubator was allowed before plating the cells on selective media.

(Composition of 2x-TSS [Transformation and Storage Solution]: 0.8 g of tryptone, 0.5 g of yeast extract, 0.5 g of NaCl, 20 g of polyethylene glycol 8000, 10 ml of dimethylsulfoxide and 10 ml of 1M $MgSO_4$-$7H_2O$, dissolve in 80 ml of water, adjust pH at approximately 6.5, autoclave and store at 4°C).

Results

I. Shotgun mutagenesis

I.1. Attempt to generate Cid$^-$ mutants by shotgun insertion-duplication mutagenesis. The general technique is illustrated in figure 7 (p. 58). Two so-called suicide vectors (pVA891 and pJCD9; (9,90)) were used. Both vectors carry an erythromycin-resistance (Erm) marker, but are unable to replicate autonomously in *S. pneumoniae*. However, Erm can be expressed in pneumococci, provided that the whole vector gets inserted in the bacterial chromosome. By themselves, the vectors cannot insert into the pneumococcal chromosome, because they lack homologous DNA. However, if fragments of pneumococcal DNA are ligated in the suicide vectors, the recombinant plasmids can successfully insert into the pneumococcal chromosome by single cross-over (Campbell-like) at the site of the homologous DNA insert (see figure 7, p. 58 and reference (89)). It is this latter property that underlies the technique of insertion-duplication mutagenesis. Only transformants with chromosomal inserts express Erm.

Potential draw-backs of the technique are also evident from figure 7. First, if the chromosomal fragment in the vector encompasses a whole gene, gene duplication (instead of insertion-inactivation) will occur. Second, if the insert contains only one end of the gene, partial duplication will restore a whole copy of the gene on one side of the insertion, but not result in gene

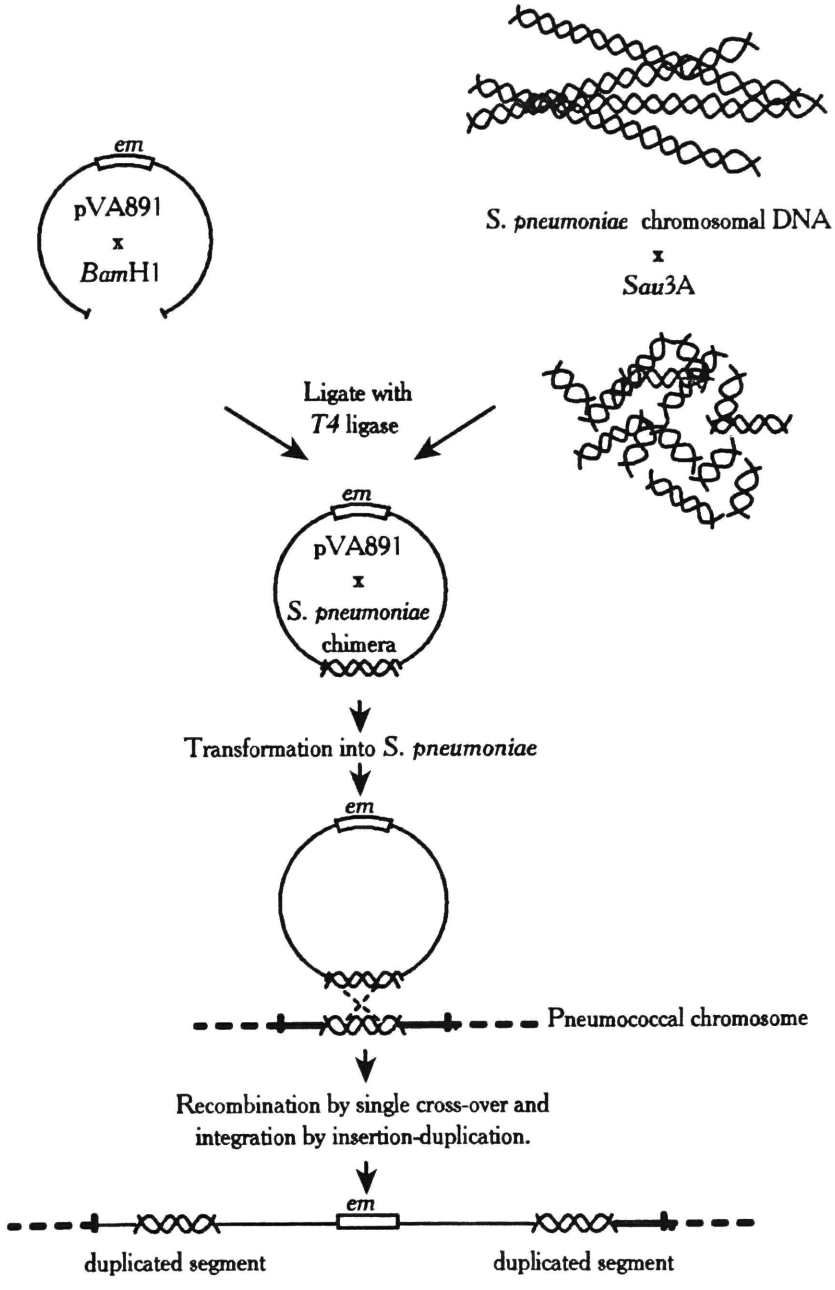


Figure 7: **The technique of insertion-duplication mutagenesis. The homologous DNA fragments ligated to the heterologous plasmid must be an internal segment of the target gene for successful gene inactivation. Larger fragments may result either in gene duplication (if the fragment encompasses the whole gene) or in restoration of one copy of the gene (if the fragment encompasses only one end of the target gene; see text for details).**

inactivation. To avoid these problems, it is critical to digest the target DNA with restriction enzymes yielding fragments smaller than the length of an average gene (i.e., less than 1 Kb).

This was achieved by digesting chromosomal DNA of the Cid$^-$ mutant Cid-1 with either of the two four base-pairs cutting enzymes *Sau3*A and *Taq*1. Cid-1 chromosome digested in this way had lost the ability to transform the *cid*$^-$ determinant into Cid$^+$ recipient cells, whereas undigested high molecular weight control DNA retained this capacity (data not shown). The *Sau3*A (resp. *Taq*1) chromosomal digests were ligated to the *BamH*1 (resp. *Cla*1) sites of either of the vectors, and the ligations were used to transform competent cells of the Cid$^+$ Lyt$^-$ mutant Lyt 4-4.

Transformants were selected for Erm (using the "sandwich" method), pooled and submitted to enrichment cycles of penicillin exposure (as described) in order to select for potential Cid$^-$ mutants. In each experiment, a positive control consisting of Lyt 4-4 transformed with high molecular weight DNA of Cid$^-$ cells was included.

In one experiment, an internal control was run to test the ability of the mutagenesis method to generate auxotrophies (using pVA891 ligated to a *Sau3*A chromosomal digest). After selection for Erm, 1000 independent transformants were tested for auxotrophy to yeast extract, by streaking each one of the colonies on Cden agar plates supplemented or not with 0.1% (w/v) of yeast extract. Yeast extract auxotrophy was chosen because it

appeared to be a characteristic of the mutant Cid-1 (see Appendix I).

Eleven out of 1000 (1%) auxotrophies to yeast extract were detected. All of them were stable, but grew slowly on rich media and were not further characterized. However, they probably had resulted from gene disruption and indicated that the method of mutagenesis worked.

The results of selection for Cid^- insertion-inactivated mutants are summarized in table 6 (p. 61). It can be seen that no Cid^- mutants could be selected out of 19,000 independent transformants that had received an insertion of the Erm marker in their chromosome. As a control, cultures of the Cid^+ Lyt^- strain Lyt 4-4 treated with high molecular weight chromosomal DNA of the Cid^- donor Cid-1 had converted to the Cid^- phenotype after only three cycles of penicillin treatment. Competent Lyt 4-4 cells transformed with closed-circular forms of either plasmids pVA891 or pJCD9 alone did not yield any Erm transformant at all.

These results suggested that either the *cid*$^-$ determinant could not be thoroughly inactivated (because null-mutants might not be viable), or that the technique of insertion-duplication mutagenesis was inappropriate to inactivate *cid*, in spite of the fact that two different four base-pairs cutting enzymes (*Sau3*A and *Taq*1) had been used and that auxotrophies could be obtained with the technique. This latter possibility led us to repeat the shotgun mutation experiment using the conjugative transposons Tn*1545* and Tn*916*.

Table 6: Attempt to generate Cid⁻ mutants by insertion-duplication shotgun mutagenesis .

Exp #	chimera		number of Erm* transf. tested	number of penicillin cycles	conversion to Cid⁻ of Erm cells	number of cycles§ to convert to Cid⁻
	suicide vector	Cid-1 digest				
1	pVA891	*Sau 3* A	1000	7	no	3
2	pVA891	*Sau 3* A	2000	7	no	3
3	pVA891	*Sau 3* A	6000	7	no	3
4	pJCD9	*Taq* 1	10000	7	no	3

* Erm transformants were pooled, grown in C+Y and cycled with 20 x the MIC of penicillin as described in chapter I.
§ Number of penicillin cycles to convert Cid^+ cultures to the Cid^- phenotype after transformation with total Cid-1 DNA.

I.2 Shotgun mutation by transposon mutagenesis. Conjugation was carried out by the filter mating method using the donor strain *E. feacalis* BM4110 for Tn*1545* and *E. feacalis* CG110 for Tn*916* (see Materials and Methods). Experiments with Tn*1545* were not pursued, because the frequency of transconjugant was too low (10^{-7}-10^{-8} per recipient cell). In contrast, experiments with Tn*916*, had a good yield (10^{-3}-10^{-4}) and were carried on. About 10,000 Tn*916* pneumococcal transconjugants were pooled and subjected to cycling with penicillin. Again, the cycling technique failed to select for Cid$^-$ Tn*916* transconjugants. In contrast, Lyt 4-4 cells (Cid$^+$ Lyt$^-$) treated with the control high molecular weight DNA of Cid-1 converted to the Cid$^-$ phenotype within only a few cycles of penicillin treatment. Thus, shotgun mutagenesis using Tn*916* also failed to generate Cid$^-$ mutants.

II. Attempt to clone the *cid*$^-$ determinant by the technique of gene conversion.

II.1. Experimental design. The experiment consisted of screening an *E. coli* library of pneumococcal DNA fragments containing the *cid*$^-$ determinant (in the plasmid vector pJCD9) for its ability to transform (by gene conversion, see figure 8, p. 64) Cid$^+$ pneumococci to the Cid$^-$ phenotype. The advantage of the technique is that (i) it does not inactivate the target gene (as in insertion-inactivation mutagenesis) and (ii), it can detect both dominant as well as recessive genes, because only one allele

is expressed at a time.

Figure 8 (p. 64) depicts the three major steps of the experimental design. First Cid-1 chromosomal DNA was digested with appropriate restriction enzymes and the smallest fragments containing the *cid*$^-$ transforming activity were isolated. Second, "mini-libraries" of these fragments were created in *E. coli*, using the cloning vector pJCD9 (9). This plasmid contains a *lacZ* polycloning site flanked by two transcription terminators, which help stabilize DNA inserts containing strong promoters, a problem that may arise when cloning pneumococcal DNA. Third, the "mini-libraries" of recombinant pJCD9 plasmids were transformed back in Cid$^+$ pneumococci and screened for their ability to transform the *cid*$^-$ determinant.

Note that the genetic recombination event expected here was a double cross-over, resulting both in conversion of the target gene and loss of the plasmid vector (see also Appendix II). This was in contrast with the technique of insertion-duplication described above (see figure 7, p. 58), in which single cross-over (Campbell-like) resulted in insertion of the plasmid and duplication of the target DNA. To promote gene conversion, DNA fragments larger than a gene ($\geq$1 Kb, resulting from digestion with 6 base-pairs cutting restriction enzymes) were ligated to the plasmid vector. In these conditions, double cross-over occurs >100x more often than single cross-over (see Pozzi et al (69) and the control experiment in Appendix II).

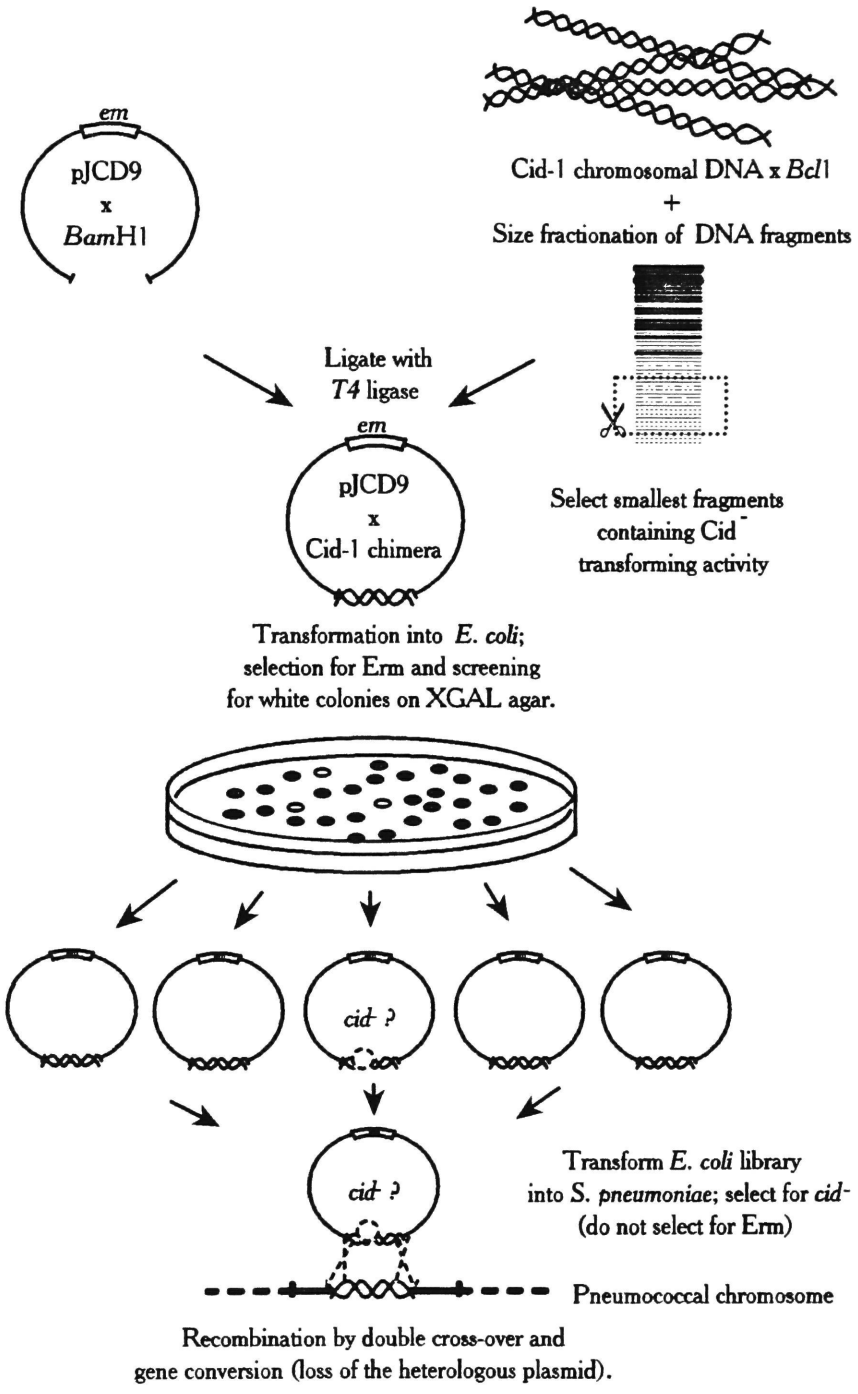


Figure 8: Experimental design used to clone the *cid*- determinant by the technique of gene conversion (see text for details).

II.2 Screening for the smallest Cid-1 DNA fragments containing the *cid*$^-$ transforming activity. Complete digestion of Cid-1 chromosomal DNA with either *Ecor*1, *Bcl*1 or *Kpn*1 did not destroy the transforming activity of the *cid*$^-$ determinant. These chromosomal digests were submitted to separation by electrophoresis on low-melting point agarose. Gel slices containing different size ranges of DNA fragments were cut, melted and used to transform Cid$^+$ Lyt$^-$ (Lyt 4-4) recipient cells to the Cid$^-$ phenotype. The *Bcl*1 digest yielded the smallest DNA fragments still containing *cid*$^-$ transforming activity (2-4-Kb), followed by *Ecor*1 (6-10 Kb) and *Kpn*1 (15-20 Kb). Therefore, *Bcl*1 was the enzyme used to digest Cid-1 DNA in further experiments.

A large scale preparation of *Bcl*1 fragments was obtained by (partial) digestion of 1000 μg of Cid-1 chromosomal DNA with the enzyme, followed by fractionation of the fragments on a sucrose gradient as described in Materials and Methods. The fraction containing the smallest DNA fragments able to transform the Cid$^-$ marker (fraction # 17, 2-6 Kb DNA fragments) was used in the next set of experiments.

C. *E. coli* "mini-libraries" of Cid$^-$ DNA followed by backcross into Cid$^+$ recipient cells. Fraction 17 was ligated to the *BamH*1 site of pJCD9 and transformed in *E. coli* DH5α. Transformants were selected for the vector marker Erm (1000 μg/ml) and screened for white colonies (containing DNA inserts) on LB-agar plates supplemented with XGal. Two thousand and four

hundred independent Erm and white transformants were isolated, grown separately in tubes containing 2 ml of LB broth supplemented with 500 μg of erythromycin/ml, and pooled in so-called "mini-libraries" of 50-70 colonies before plasmid DNA was extracted. Heterogeneity of the plasmid sizes was controlled by agarose gel electrophoresis. These "mini-libraries" containing the putative *cid*$^-$ gene were then transformed in the Cid$^+$ Lyt$^-$ pneumococcal recipient Lyt 4-4. Selection was <u>not</u> for Erm, (which would have accounted for insertion of the plasmid, as described above), but for the Cid$^-$ phenotype, by cycling with penicillin.

A positive control tube received high molecular weight DNA of the Cid$^-$ mutant Cid-1, and a negative control received DNA of the wild-type Cid$^+$ Lyt$^+$ strain R6xStr carrying a streptomycin-resistant marker to account for the overall frequency of transformation (which was 0.1-1%). In addition, in certain experiments, an internal control was run by subculturing aliquots of transformed cells on erythromycin-containing plates to test for the ability of the recombinant plasmids to insert into the chromosome by single cross-over (see above). Such Erm transformants occurred at a frequency of 0.01%, demonstrating that passage through *E. coli* was not incompatible with DNA processing by pneumococci.

The results are summarized in table 7 (p. 67). While high molecular weight DNA of the mutant Cid-1 as well as the *Bcl*1 digest fraction 17 (used as target fragments to clone in pJCD9), transformed Cid$^+$ recipient cells to the Cid$^-$ phenotype, neither

Table 7: **Testing *E. coli* "mini-libraries" of Cid-1 DNA for transformation of the Cid⁻ phenotype.**

	Penicillin cycling		
Source of DNA	number of cycles to select Cid⁻	total numbers of cycles	conversion to Cid⁻ phenotype
R6x chromosome	> 8	8	No
Cid-1chromosome	3-4	8	Yes
Bcl 1 Fract 17	4-5	8	Yes
§"Mini-libraries"			
# 1	> 8	8	No
# 2	> 8	8	No
# 3	> 8	8	No
# 14	> 8	8	No

§ = fraction 17 of sucrose density gradient of Cid-1 x *Bcl* 1 digest.
(see text for details)

of the 14 "mini-libraries" succeeded in converting Cid^+ cultures to the Cid^- phenotype.

Thus, cloning of the *cid*$^-$ marker using *E. coli* DH5α as an intermediate host did not succeed, in spite of the fact that (i) successful gene conversion could be achieved in pneumococci with the experimental system described (see Appendix II), (ii) the *Bcl*1 pneumococcal fragments ligated to pJCD9 transformed the *cid*$^-$ determinant into pneumococci, (iii) adequate quality and quantities of the "mini-libraries" DNA were used to transform pneumococci (as shown by the internal control described above) and (iv), theoretically enough Erm $LacZ^-$ *E. coli* recombinants were picked to assure the adequate representation of all the *Bcl*1 pneumococcal fragments present in fraction 17.

These results raised the possibility that the *cid*$^-$ marker might have been lost during passage through the *E. coli* host, either because it was incompatible with plasmid maintenance, or because its product was toxic to the cells.

Discussion

The selection for Cid$^-$ mutants by gene insertion inactivation mutagenesis did not fail for trivial reasons. Both techniques of mutagenesis described above have been successfully used to interrupt several genes both in pneumococci and other streptococcal strains (59,91). For instance, insertion-duplication mutagenesis was used by Morrison et al. (59) and Masure et al. (personal communication) to interrupt and clone pneumococcal genes involved in competence. In both cases, pneumococcal chromosome was digested with the 4 base-pair cutting enzyme *Sau3*A before being ligated in adequate vectors and transformed back into pneumococcal recipient cells. Effective inactivation of target genes (affecting competence) were found in about 1/1000 insertion-transformants. Considering that competence involves probably $\geq$ 10 genes (up to 30 proteins seem to be involved in the whole process of transformation; (59)) their chance to target one specific gene was not more than 1/10,000 insertions. In our experiments, we used two types of 4 base-pair cutting enzymes (*Sau3*A and *Taq*1) and obtained 1/100 auxotrophies to yeast extract. This relatively high number of hits (as compared to 1/1000 in Morrison's experiments) might have several explanations, including the possibility of redundant insertions in the same gene and/or the fact that yeast extract auxotrophy might involve many more genes than competence. However, the results of this control experiment showed that the technique

worked, and the absence Cid$^-$ mutants among the 19,000 insertion-transformants screened (10-20x more than Morrison et al. or Masure) arose the possibility that either (i) mutants with insertion-inactivated *cid* gene were not viable or (ii), the *cid* gene could not be inactivated because it did not have two internal restriction sites for the enzymes used (see figure 7, p. 58).

The failure to obtain Cid$^-$ mutants using Tn*916* insertion mutagenesis is an argument for the former possibility. Tn*916* has been successfully used to interrupt genes in both pneumococci and group B streptococci (73,91). On the other hand, insertion of Tn*916* might not be perfectly random in pneumococci, because its mechanism of insertion involves a circular intermediate with an AT-rich repeat around the insertion site (10), which might affect the randomness of insertion into AT-rich target DNA (such as the pneumococcal chromosome). Therefore, as with insertion-duplication mutagenesis, one cannot formally exclude that the failure to select for insertion-inactivated Cid$^-$ mutants was not due to the lack of correct hit by Tn*916*.

However, the number of negative experiments and the fact that both techniques had been successful in interrupting genes other than *cid* are indirect arguments for the fact that null-*cid* mutants might not be viable. An instructive precedent for such a hypothesis is that transposon-mutagenesis also failed to yield Cid$^-$ mutants in *Staphylococcus aureus* (Oshida personal communication). In these experiments, a Cid$^+$ staphylococcal

strain was mutagenized with Tn*551*. This transposon has been successful in interrupting a number of genes related to methicillin-resistance, including the gene of the penicillin binding protein 2A (PBP 2A) and several so-called auxiliary genes responsible of the control of peptidoglycan synthesis (5,53,62). When libraries of Tn*551*-insertion mutants of staphylococci were cycled with high concentrations of methicillin, Cid$^-$ derivatives arose, as in pneumococci. However, the Cid$^-$ phenotype did not cotransform with the transposon in backcross experiments, indicating that the Cid$^-$ phenotype was not due to the transposon, but to another (spontaneous) mutation located elsewhere in the chromosome. Thus, as in pneumococci, null mutants in the "*cid*" determinant might not be viable in staphylococci.

For this reason, we attempted to clone the *cid*$^-$ determinant by gene conversion. Control experiments in which the *lytA::em* of RUP-24 was converted to *lytA* by the *E. coli* plasmid pGL80 (containing the cloned pneumococcal *lytA* gene) confirmed that the experimental design was appropriate (see Appendix II). In addition, the *E. coli* cloning vector (pJCD9, (9)) used to expand pneumococcal DNA beared two transcription terminators flanking the cloning site, to decrease the risk of instability of recombinant plasmids containing DNA inserts with strong promoters. In spite of these precautions, the *cid*$^-$ transforming activity was lost during passage through *E. coli*. This was unlikely to be due to a trivial misrepresentation of the correct DNA fragment in the library, since the library had been enriched

in DNA fragments containing the *cid*$^-$ marker and care had been taken to collect a large number of recombinant clones (2400). In addition, each recombinant clone had been grown separately to turbidity before being pooled for plasmid extraction. Thus, the most likely explanation was that the plasmid containing the *cid*$^-$ determinant could not be maintained in *E. coli*.

The frequency of *E. coli* "persisters" (i.e., survivors) during ampicillin treatment has been shown to be under the control of a gene called *hypA* (for high ampicillin persisters), which has been cloned (60,61,75). The gene encodes for a ca. 50,000 kD protein that might be involved in the regulation of cell division. Most interestingly, *hypA* could not be maintained in a plasmid under the control of a strong promoter, presumably because its product is toxic for the cell (61). If *cid*$^-$ performs some equivalent function to *hipA*, it is conceivable that cloning the *cid*$^-$ determinant in a multicopy plasmid might also result in toxicity for *E. coli*. Whether *cid*$^-$ is the pneumococcal equivalent of *hypA* is not known. However, both Cid$^-$ and HipA phenotypes share the ability to reduce killing due to inhibition of either cell wall synthesis (beta-lactams) or DNA replication (quinolones; *cid*$^-$ decreased temofloxacin-induced killing by ca. 100x in pneumococci, data not presented).

As envisioned above, it is possible that the *cid* determinant is a gene that performs some vital function that can neither be completely eliminated from the cell, nor overexpressed. The appearance of a new nutritional requirement - genetically

inseparable from the *cid* locus - is also consistent with this notion (see Appendix I). Moreover, since *cid*$^-$, as *hypA*, appears to control cell death due to both inhibition of cell wall synthesis and DNA replication (two events that are synchronized with cell division; (32)), it is possible that the *cid* product might be directly of indirectly involved in the regulation of cell division, as has been suggested for the *hypA* determinant of *E. coli* (75).

Chapter III

Studies on the biochemistry and physiology of cell wall degradation in Cid$^+$ and Cid$^-$ pneumococci.

Summary of results

The experiments presented in the first chapter have suggested the existence of (at least) two consecutive events during penicillin-induced killing and lysis of pneumococci: first killing and second lysis. It was shown that killing and lysis were formally separate phenomena that could be dissociated in both physiological experiments (during penicillin treatment of Lyt$^+$ and Lyt$^-$ mutants) and in genetic crosses, because killing and lysis could be affected separately by independent mutations (resulting in Cid$^-$ and/or Lyt$^-$ phenotypes). However, while the major mechanism(s) of penicillin-induced killing of pneumococci appeared to be autolysis-independent, neither the nature of the lethal event, nor the mechanism of protection afforded by the *cid*$^-$ mutation against penicillin-induced killing and lysis were specified. Therefore, it was important to run complementary biochemical and physiological experiments, in order to better define both the cellular localization of the major (autolytic-independent) killing event and the mechanism of protection afforded by the *cid*$^-$ mutation.

Figure 9 (p. 76) depicts the principal known penicillin targets of the cells. The primary targets are the membrane-bound penicillin-binding proteins (PBP), which are "irreversibly" acylated and inactivated by the antibiotic. The secondary targets are the autolytic enzymes and their substrate, the bacterial cell wall peptidoglycan, which are responsible for cell wall degradation and bacterial lysis during penicillin treatment. Additional molecules also known to influence bacterial autolysis and present in these structures are depicted in figure 9: they include the cell wall-bound teichoic acids and the membrane-bound lipoteichoic acids (20,82).

In order to detect potential differences between these cellular structures in Cid^+ and Cid^- cells, we have run a step by step biochemical analysis of some specific properties of these penicillin targets in both types of bacteria. In addition, physiological experiments have allowed us to more accurately define the cellular level at which the *cid*$^-$ mutation might operate to protect pneumococci from penicillin-induced killing.

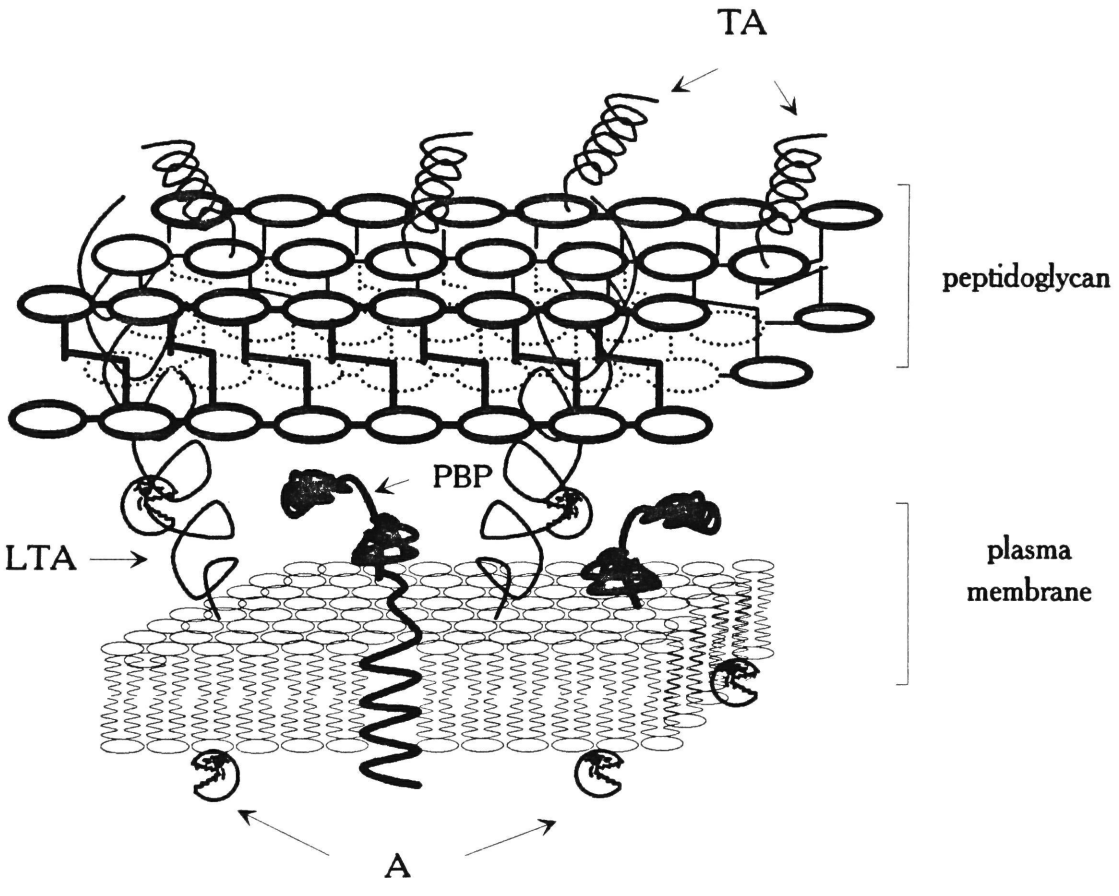


Figure 9: **The cartoon depicts the most common primary and secondary penicillin targets of pneumococci. PBP = penicillin-binding proteins (primary targets); A = autolysins (secondary targets); LTA = lipoteichoic acids (involved in the regulation of autolysis); TA = cell wall teichoic acids (absolutely required for the activity of amidase in peumococci).**

Materials and Methods

Titration of the penicillin-binding proteins (PBPs). Exponential-phase microorganisms (1 ml; ca. 8 x 10^7 cells/ml) were incubated with [^{3}H]-benzylpenicillin at concentrations ranging from 0.3 to 10x the MIC at 37°C for 10 min. An excess of unlabeled benzylpenicillin (1.7 mM) was then added, and the samples were immediately chilled on ice. The bacteria were recovered by centrifugation (12,000 x g for 5 min at 4°C), suspended in 50 μl of 0.05 M sodium phosphate buffer (pH 7.0) containing 1.7 mM of unlabeled benzylpenicillin and 1% Sarkosyl NL-97 (Sigma), and incubated for 5 min at 37°C. This treatment resulted in complete lysis of the microorganisms and inactivation of the PBPs. The lysates were then prepared for SDS-PAGE. The techniques used for discontinuous gel electrophoresis, staining, and detection of the PBPs by fluorography have been described earlier (93).

Cell wall preparation. Pneumococcal cell walls were prepared by a published procedure (18,20). In brief, exponentially growing cultures were rapidly chilled by immersing the flasks in an ethanol-ice bath until the temperature reached 4 to 0°C. After the cells were harvested by centrifugation at 4°C, they were suspended in iced phosphate-buffered saline and quickly dropped into boiling SDS (4% final concentration) to inactivate any wall-modifying enzymes. Walls were mechanically broken by

shaking with acid-washed glass beads in a Vortex mixer operating at top speed. It took about ten 1-min pulses to disrupt over 90% of the microscopically identifiable bacterial forms. Unbroken cells and glass debris were sedimented by low-speed centrifugation (5,000 x g, 10 min). Cell wall fragments were pelleted at 25,000 x g, suspended in buffered saline with 0.05% sodium azide, and digested with DNase, RNase, and protease as described, except that proteinase K was substituted for trypsin. Peptides from protease digestion and remaining lipids were extracted with boiling 1% SDS, and the wall fragments were washed twice with water and incubated for 15 min at 37°C, first with 8 M LiCl and then with 100 mM EDTA, to remove material bound by ionic interactions. After another water wash the fragments were treated with acetone, suspended in water, and lyophilized.

To introduce a radioactive label into the cell walls, one of the following methods was used. (i) For [^{3}H]-lysine labeled walls, bacteria were grown for 2 h in the chemically defined medium Cden containing L-[4,5-^{3}H(N)]-lysine (New England Nuclear Corp., Boston, MA) at a final concentration of 5 uCi and 10 μg/ml. (ii) For [^{3}H]-choline labeled walls, bacteria were grown in the semi-synthetic medium C+Y, to which [methyl-^{3}H]-choline chloride (New England Nuclear Corp.) was added at a final concentration of 1 uCi or approximately 15 ng/ml.

Preparation of cell extracts and measurement of their cell wall-hydrolyzing capacities.

(i) For extracts of Lyt⁺ pneumococci: 10 ml cultures (in C+Y) were grown to an OD_{620} of 0.7, chilled, harvested by centrifugation, washed and resuspended in 500 µl of 50 mM Tris maleate buffer at pH 7 + 0.1% (v/v) of the detergent Brij 35. The cells were lysed by the addition of 0.1% of DOC (w/v, final concentration) followed by incubation for 10 min at 37°C. The lysates were then chilled and "cleared" by centrifugation for 5 min at 10,000 x g (at 4°C). Protein concentration was determined in the supernatant (using the bicinchoninic method (75A)) and the samples were frozen at -70°C.

To compare the cell wall-hydrolyzing capacity of the extracts, 400 µg (in 200 µl of buffer) of proteins were 2x-serially diluted in 8 tubes (from 1/1 to 1/128 plus one negative control) and 25 µl of each dilution (plus the control) was transferred to a series of tubes containing 155 µl of the same buffer + 20 µl of [^{3}H]-lysine labeled pneumococcal cell walls (specific activity 3 x 10^5 cpm; 660 µg/ml). The tubes were incubated for 30 min at 37°C, after which time the reaction was stopped by rapid chilling and addition of 25 µl of formaldehyde and 25 µl of 1% bovine serum albumin. After centrifugation for 10 min at 10,000 x g, the radioactivity that was released to the supernatant was measured by liquid scintillation counting.

(ii) For Lyt⁻ pneumococci: Extracts of pneumococci from which the genetic determinant of the major autolysin (amidase) was deleted contained another enzymatic activity capable of a slow hydrolysis of cell walls (17,74). To prepare enzyme extracts from Lyt⁻ cells, bacterial cultures (1 liter) were harvested in the middle of the exponential growth phase (about 0.5-1 x 10^8 CFU/ml). Pellets were suspended in 5 ml of 10 mM Tris hydrochloride (pH 6.8) containing 10 mM $MgCl_2$ and DNase I and RNase I, each at 100 μg/ml; the suspension was mixed with an equal volume of glass beads (diameter, 100 μm; Thomas Scientific, Swedesboro, NJ) and stirred vigorously by vortexing (Scientific Industries Inc., Bohemia, NY) at 4°C for 10 to 15 min. The detergent Brij 35 and a LiCl solution were added to give 0.1% and 1.0 M final concentrations, respectively. The debris of broken cells and glass beads was removed by centrifugation (10,000 x g for 10 min), and the supernatant was further clarified by ultracentrifugation at 40,000 x g for 1 h. The supernatant was then dialyzed overnight at 4°C against 10 mM Tris hydrochloride (pH 7.0) containing 0.1% Brij 35, and small batches containing 1.0 mg of protein per ml were stored frozen at -70°C.

The enzymatic activity in these extracts was determined as follows. A total of 50 μl of enzyme extract (protein concentration 1 mg/ml) was mixed with 90 μl of buffer (10 mM Tris hydrochloride [pH 6.8]), 10 μl of Brij 35 (5% solution), and 20 μl of [^{3}H]-lysine-labeled cell walls, and the suspension was incubated for several hours at either 30 or 37°C. The reaction

was stopped and the radioactivity that was released to the supernatant was measured as described above. For the removal of cell wall teichoic acids, cell walls (3.6 mg) were suspended in 2 ml of 49% hydrofluoric acid (HF), and the suspension was stirred slowly at 0°C for 24 h. The HF was removed by centrifugation of the cell walls and extensive washing of the pellet with distilled water. The pellet (cell wall peptidoglycan free of teichoic acid) was dried (recovery, 1.6 mg) and subsequently suspended in distilled water and stored at -20°C. This treatment quantitatively removed teichoic acid components of the cell wall, as indicated by chemical analysis for galactosamine, phosphorus, and choline.

Cell wall hydrolysis by purified amidase. Purified amidase was prepared by a previously described method (20) from cultures of the *E. coli* strain CM21 (14) containing the cloned pneumococcal *lytA* gene in the plasmid pGL80. One unit of amidase activity was defined as the amount of enzyme capable of digesting 1 ug of cell wall in 10 min). Susceptibility of various cell wall preparations to hydrolysis by purified amidase *in vitro* was titrated by a dilution method similar to that described for Lyt^+ cell crude extracts (see above), except that purified amidase was used as an enzyme and various [^{3}H]-choline labeled cell walls were used as substrates (see Results section).

Results

I The primary penicillin target: the penicillin binding proteins (PBP). Pneumococci contain five high molecular weight penicillin binding proteins (PBP 1a, 1b, 2a, 2b and 3 resp.; (93)), which can be covalently labeled with radioactive penicillin and visualized by fluorography after separation by SDS-PAGE. In the present experiments, Cid^+ pneumococci and the Cid^- mutant Cid-1 contained a similar set of PBPs, as measured both by their migration on SDS-PAGE and as well as by their affinity for radioactive penicillin (see figure 31, appendix VII, p.154).

II The secondary penicillin target: the cell wall and the autolytic enzymes.

II.1 Susceptibility of Cid^+ and Cid^- purified cell walls to digestion by the major autolysin amidase.

(i) The walls of untreated pneumococci: We have tested the ability of purified amidase to solubilize continuously radiolabeled (with [^{3}H]-choline) purified cell walls prepared from growing cultures of either Cid^+ Lyt^- (Lyt 4-4) or Cid^- Lyt^- (T6) cells. Equal amounts of radiolabeled cell walls were distributed into pre-chilled Eppendorf tubes containing increasing amounts of amidase (from about 0.4 to 50 U/ml). The

tubes were then incubated at 37°C for 20 min, after which the reaction was stopped as described. The undissolved cell wall material was pelleted and the radioactivity released to the supernatant (solubilized wall) was counted in a scintillation counter. The fraction of solubilized wall in each tubes was expressed as the percent of the total radioactivity that was released to the supernatant. Figure 10 C (p. 84) shows that the titration curves of Cid^+ and Cid^- walls were parallel, indicating that in these conditions both types of cell walls were solubilized at the same speed by purified amidase.

(ii) The "newly-made" wall in penicillin-treated pneumococci. It is known that even after addition of penicillin to the cultures, pneumococci can still synthesize some residual cell wall for some time (94). Therefore, it was possible that in Cid^+ cells, such "newly-made" wall might be more susceptible to digestion by amidase than in Cid^- cells. To test this hypothesis, growing cultures of either Lyt 4-4 (Cid^+ Lyt^-) or T6 (Cid^- Lyt^-) received 20x the MIC of penicillin and, immediately after addition of the drug, were labeled for 2 h with [^{3}H]-choline, until the experiment was stopped for cell wall purification. Figure 10 D (p. 84) shows that again, both types of "newly-made" walls were solubilized at virtually identical rate by amidase *in vitro*. Thus, purified cell walls of either Cid^+ of Cid^- pneumococci were equally susceptible to digestion by purified amidase, whether or not the cells had been treated with

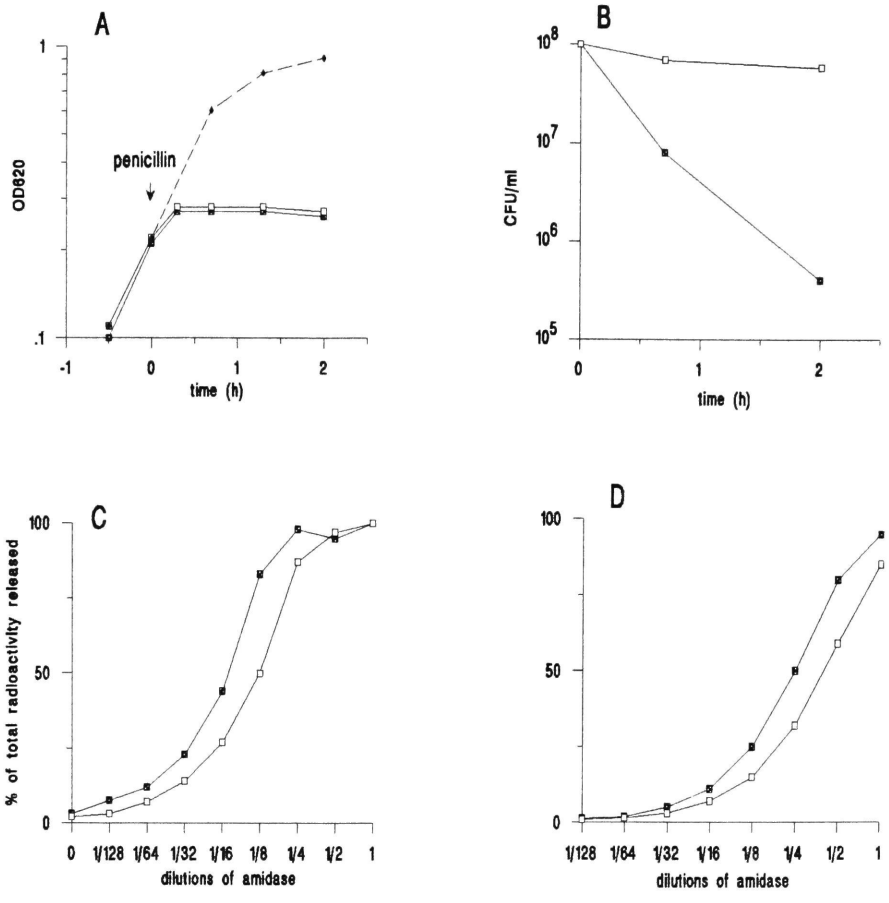


Figure 10: **Time-kill curves and titration of Lyt 4-4 (Cid^+ Lyt^-; closed symbols) and T6 (Cid^- Lyt^-; open symbols) cell wall degradation by amidase *in vitro.* At time zero (arrow in panel A), 20x the MIC of penicillin was added to the cultures and OD_{620} (A) and bacterial survival (B) were followed. Panels (C) and (D) depict the susceptibility to digestion by amidase of either globally radiolabeled cell walls (C) or "newly made" walls (D), which had been pulse-labeled for 2 h with [^{3}H]-choline <u>after</u> the addition of penicillin to the cultures. The dashed line in panel A shows the growth of untreated control cultures.

penicillin prior to wall purification, and whether or not the cells had died as a consequence of antibiotic treatment.

II.2 Structural analysis of Cid$^+$ and Cid$^-$ pneumococcal walls. The lack of difference between the susceptibility of Cid$^+$ and Cid$^-$ cell walls to amidase digestion was further substantiated by the absence of major differences in the structures of these walls as analyzed by two biochemical techniques. First, the patterns of peptide crosslinks between the stem peptides of both types of peptidoglycans appeared to be identical, as shown by analysis of wall hydrolysates by high performance liquid chromatography (HPLC) (see Appendix III). Second, the proportion of cell wall subunits bearing teichoic acids was also similar in both types of cells, as determined by separation of "teichoicated" from "non-teichoicated" cell wall subunits by affinity chromatography (20) (data not presented).

Thus, at least on the level of resolution allowed by the analytical techniques, functional or structural differences in cell walls were apparently not responsible for the different behavior of Cid$^+$ and Cid$^-$ cells during penicillin treatment. In the next set of experiments, we have sought possible differences between the levels of autolytic enzymes in both types of bacteria.

II.3 Activity of autolytic enzymes in extracts of Cid$^+$ and Cid$^-$ cells.

(i) Specific activity of amidase. We have already shown that both the wild-type Cid$^+$ Lyt$^+$ strain R6x and the Cid$^-$ Lyt$^+$ mutant Cid-1 produced qualitatively and quantitatively equal amounts of autolytic amidase, as measured by enzymatic activity in cell extracts (see chapter I, tables 3-5, p. 40, 43 and 45 resp.).

(ii) Specific activity of a second wall acting hydrolase in Cid$^+$ and Cid$^-$ cells. In addition to the major autolysin amidase, pneumococci contain a second enzyme the primary activity of which appears to introduce breaks ("nicks") into the glycan chain of the cell wall (17,74). A penicillin-induced abnormal activity of such an enzyme could contribute to the killing effect of this antibiotic. We compared the specific activities of this second cell wall acting hydrolase in cell extracts prepared from Cid$^+$ Lyt$^-$ (Lyt 4-4) and Cid$^-$ Lyt$^-$ (T6) pneumococci. Table 8 (p. 87) shows that no difference was detected.

In an additional set of experiments (see Appendix IV) we have further investigated the importance of such second "nicking" murein hydrolase activity in penicillin-induced killing of pneumococci. A new type of mutant (temporarily called Lys-2), which overproduced such a "nicking" enzyme was generated by insertion-duplication mutagenesis, with the suicide vector pVA891. When transformed into Lyt$^-$ cells (either Lyt 4-4 or a

Table 8: Specific cell wall-hydrolysing activities in extracts of isogenic Cid$^+$ and Cid$^-$ pneumococci

Enzyme source[a]	Cell wall substrate[b]	cell wall material released (%) at[c]:	
		37°C	30°C
Cid$^+$ Lyt$^-$	complete wall	1.6	3.6
Cid$^-$ Lyt$^-$	complete wall	1.5	3.5
Cid$^+$ Lyt$^-$	HF-stripped wall[d]		8.12
Cid$^-$ Lyt$^-$	HF-stripped wall[d]		7.85

[a] Crude extracts were prepared as described in the text. Protein concentrations were 1 mg/ml for Lyt$^-$ Cid$^+$ pneumococci and 1.1 mg/ml for Lyt$^-$ Cid$^-$ pneumococci.

[b] Cell walls that was biosynthetically labeled with radioactive lysine were prepared from a Lyt$^+$ Cid$^+$ strain (R6x; see text for details).

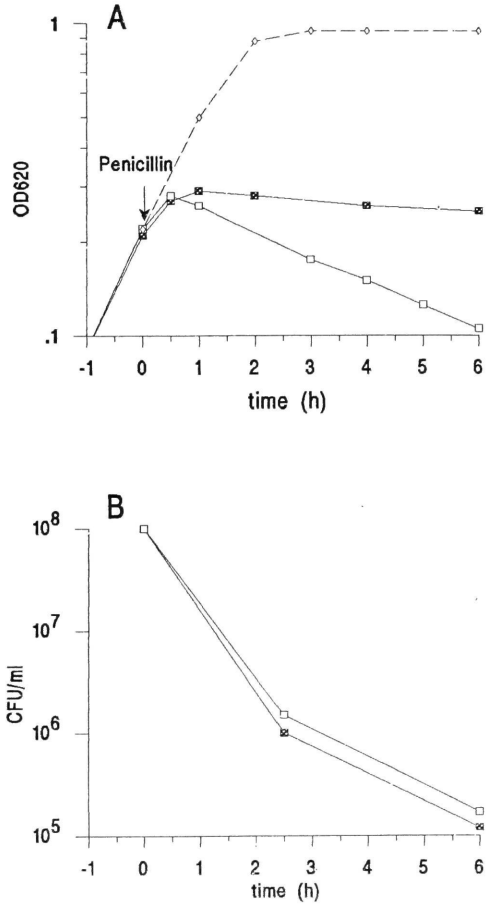
[c] Hydrolysis of cell walls were measured after 18 h of incubation, as described in the text.

[d] Cell walls were treated HF to remove teichoic acid (see text).

double insertion-inactivated mutant (*lytA::em::cm*), the Lys-2 mutation produced a consistent increase in the rate of penicillin-induced lysis (see figure 11, p. 89), which was not due to the major autolysin amidase, but resulted from the introduction of breaks in the cell walls (see inset in figure 11). In the $Cid^{-}Lyt^{-}$ mutant T6, lysis was also increased by Lys-2. These wall lesions were genuine "nicks", because they were not accompanied by cell wall solubilization. However, while the Lys-2 mutant overproduced a "nicking" enzyme that might be the analog of the "second" pneumococcal murein hydrolase described by Lopez et al (17,74), the mutation did not increase the rates of penicillin-induced killing in either Cid^{+} or Cid^{-} pneumococcal transformants (see figure 11 bottom panel).

Thus, as with cell wall susceptibility to amidase *in vitro*, no differences were found in the levels of either one of the two known autolysins between Cid^{+} and Cid^{-} cells. Therefore, the decreased penicillin-induced lysis observed in the Cid^{-} Lyt^{+} mutant Cid-1 may be mediated by a completely different mechanism, possibly through limitation (regulation) of the access of autolytic enzymes to their substrate, the cell wall. This hypothesis was tested in the experiments to be described next.

Figure 11: **Cultures of either Lyt 4-4 (Lyt$^-$; closed symbols) or the Lys-2 mutant overproducing a nicking enzyme (open symbols) were treated with 20x the MIC of penicillin (arrow) and OD_{620} (panel A) and viable counts (panel B) were followed. The insets in panel A show that the Lys-2 mutant accumulated substantially more breaks or "nicks" (arrows) in the cell wall than the control strain Lyt 4-4 during penicillin treatment. These "nicks" were not accompanied by cell wall solubilization as Lys-2 cultures radiolabeled in their cell walls did not release radioactivity during penicillin-induced lysis (see appendix IV). The dashed line in panel A depicts the growth of untreated control cultures.**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1
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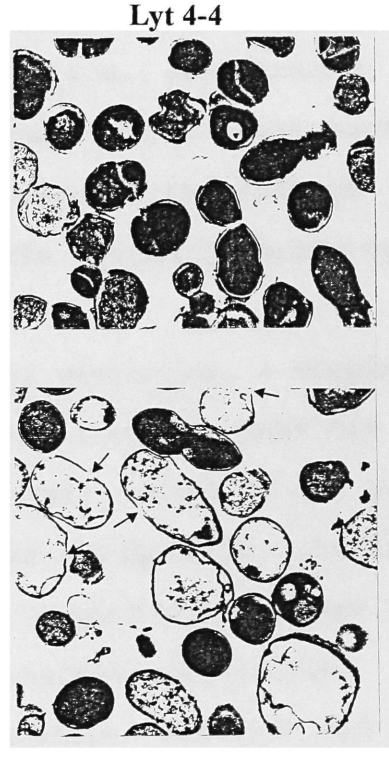
Lyt 4-4
Lys-2

III. Physiological investigations on penicillin-induced killing and lysis of Cid$^+$ and Cid$^-$ pneumococci.

III.1 Access of amidase to its cell wall substrate in the *in vivo* context. As suggested above, the *cid*$^-$ mutation might control penicillin-induced lysis of Lyt$^+$ cells by limiting the access of the enzyme to its cell wall substrate. Since amidase is constitutively produced in Lyt$^+$ pneumococci (i.e., pneumococci that bear a functional *lytA* gene; (12)), such control must occur at the post-translational level, anywhere between the step of membrane export to that of regulation of the enzymatic activity *in situ* (i.e., within the cell wall).

In order to better define this control mechanism, a simple experiment was devised. The system relied on the fact that the physiological transport of amidase to the wall (which is not yet understood) could be bypassed by adding the enzyme from outside the cell. It has been shown that addition of purified amidase to cultures of Lyt$^-$ pneumococci could fully restore penicillin-induced lysis in these bacteria (83). Therefore, Cid$^+$ Lyt$^-$ and Cid$^-$ Lyt$^-$ pneumococcal cultures supplemented or not with purified (exogenous) amidase were exposed to 20x the MIC of penicillin, and both survival and OD_{620} were followed.

Figure 12 A and C (p. 92) shows that addition of exogenous amidase to Cid$^+$ Lyt$^-$ (Lyt 4-4) cultures restored wild-type penicillin-induced lysis (as expected), while it had only marginal influence on bacterial survival. This was in accordance

with previous observations showing that, lysis was not the primary killing factor during penicillin treatment of pneumococci.

In contrast, however, penicillin-treated cultures of Cid$^-$ Lyt$^-$ cells were very poorly lysed by exogenous amidase and killing was not increased by the enzyme either (figure 12 B and D, p. 92)). Thus, when assembled back in its *in vivo* context, the wall of Cid$^-$ cells could barely be lysed by exogenous amidase, in spite of the fact that it was fully sensitive to amidase degradation *in vitro*. This discrepancy supported the hypothesis that the autolytic system was controlled by an alternative element, that was fully expressed in whole cells and was involved in the regulation of both penicillin-induced killing and lysis.

A clue for the cellular localization of such element was provided by the observation that Cid$^-$ cells were rapidly lysed by treatment of the cultures with the detergent DOC (figure 12 B, p. 92). Since detergents primarily alter the stability of the cellular membrane, it is likely that the common target controlling both penicillin-induced killing and lysis operates at the level of the plasma membrane. A model for such an element is presented in the discussion (see also figure 13, p. 99).

III.2 Additional investigations on the nature of penicillin-induced killing. Two additional sets of experiments were performed to better specify the nature of the bactericidal lesion of penicillin in the cell (see Appendixes V and VI).

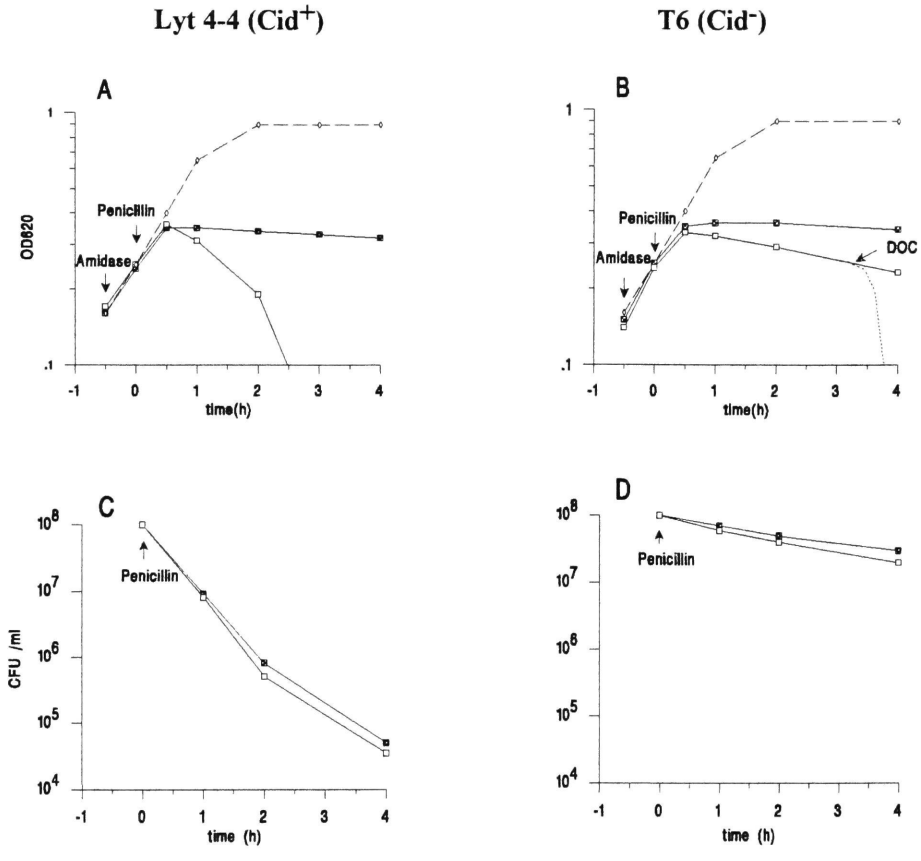

Figure 12: **The *cid*⁻ mutation interferes with the access of amidase to its cell wall substrate. Cultures of both Lyt 4-4 (Cid^+ Lyt^-; panels A and C) and T6 (Cid^- Lyt^-; panels B and D) received amidase (25 U/ml; closed squares) or no enzyme (open squares) 30 min before treatment with 20x the MIC of penicillin (arrow). Untreated control cultures (dashed line) were not affected by addition of amidase. Optical density and viable counts were followed over time. Note that addition of DOC to the Cid^- mutant T6 fully restored lysis by amidase (see dotted line in panel B).**

(i) Penicillin-induced degradation of bacterial RNA. In group A streptococci, which are exquisitely sensitive to the bactericidal effect of penicillin, while notably resistant to antibiotic-induced lysis (27,55), penicillin-induced killing has been associated with intracellular degradation of RNA (56). It was suggested that the cidal effect of the drug might be mediated by some antibiotic-induced activity of endogenous nucleases. We have tested whether degradation of radiolabeled RNA might be different in penicillin-treated Cid^+ and Cid^- pneumococci (see Appendix V). No difference was observed.

(ii) Survival of penicillin-treated pneumococci in osmotically-protective medium. Experiments in which Cid^+ Lyt^+ (R6x) and Cid^+ Lyt^- (Lyt 4-4) pneumococcal strains were grown and treated with penicillin in osmotically-protective medium (containing 30% of raffinose and 20 mM of Mg^{++}) are described in Appendix VI. Adequate conditions permitted the conversion of penicillin-treated Lyt^+ bacteria to stable protoplasts. As expected, similar treatment of Lyt^- cells did not induce alteration of the bacterial shape. Surprisingly, however, in spite of the fact that the medium provided osmotic protection and also supported bacterial growth, penicillin treatment resulted in identical bacterial killing, whether or not the cultures had been grown in osmotically-protective media. Therefore, in these experiments, protection against osmotic imbalance did not prevent penicillin-induced killing of pneumococci.

Discussion

Taken together, these results support the hypothesis presented in chapter I, that *cid* controls both penicillin-induced killing and lysis by altering the function of a "new" penicillin target. Indeed, both functional and structural analysis of the known primary and secondary penicillin targets of pneumococci showed no major differences between Cid^+ and Cid^- cells. First, both types of pneumococci contained a "normal" set of PBPs (as determined by migration on SDS-PAGE) with similar affinity for penicillin, which was also consistent with the unchanged MIC value of the drug for Cid^- as compared to Cid^+ bacteria. Second, neither the composition of cell walls nor the levels of two autolytic enzymes seemed to differ in Cid^+ and Cid^- pneumococci when tested *in vitro*. Therefore, the cid^- mutation was likely to operate at the level of some other target(s), only functionally linked to the "classical" penicillin targets of bacteria.

A clue for the physical localization of the Cid^- alteration came from the experiments in which exogenous amidase was added to Lyt^- pneumococcal cultures prior to penicillin treatment. This reconstitution experiment allowed to challenge the effect of the cid^- mutation in a system where the wall was in its "*in vivo*" context, but the transport of amidase through the plasma membrane was bypassed. Unexpectedly, the cid^- mutation was also able to control penicillin-induced lysis in these conditions. Importantly, however, instantaneous cell lysis was restored by

treatment of the cultures with the detergent DOC. This indicated that alteration of some lipophilic structure in the bacteria could nullify the protective effect of *cid*$^-$ on amidase-induced lysis. The major structure to be altered by such treatment is obviously the plasma membrane.

The implication of the plasma membrane in the regulation of autolysis has long been recognized (82). However, the mechanism(s) by which membrane molecules regulate autolysis is incompletely understood. For instance, the transport of the pneumococcal amidase and other lytic enzymes (including bacteriophage-encoded lysins) across the plasma membrane remains an enigma (12). Since neither the *lytA* gene, nor the genes of known phage lysins encode for a signal sequence, the enzymes are likely to use some host or phage-encoded channel proteins (to be referred to as "holin" in accordance with the proposition of Young (96)) to reach their cell wall substrate. However, such autolysin-transport holins have yet to be isolated in pneumococci.

Once across the membrane, the autolysin amidase becomes anchored to the plasma membrane through the choline-containing (membrane-bound) lipoteichoic acids or LTA (3,35,82). LTA are unique glycolipid-linked pneumococcal teichoic acids, which are strong inhibitors of amidase. In conditions that promote cell lysis, LTA are released to the supernatant (3,82). Experimental evidence suggests that during that process, amidase molecules attached to the choline residue of the LTA might progressively

exchange for the choline residue of the pneumococcal cell wall teichoic acids (which typically contain choline), allowing the enzyme to be properly positioned in the peptidoglycan and hydrolysis of the cell wall to proceed (51). Since LTA molecules are membrane-bound glycolipids, their release must involve some disturbance(s) of the plasma membrane. However, the factors triggering the release of LTA molecules from the membrane are not known.

While it is conceivable that the protective effect afforded by *cid*$^-$ was due to some sophisticated change in the cell wall structure of Cid$^-$ cells, this is unlikely because Cid$^-$ Lyt$^+$ cells were lysed normally by DOC and because amidase can rapidly and completely degrade cell walls prepared from Cid$^-$ Lyt$^-$ cells. We favor an alternative model, in which the *cid* product operates at the level of the plasma membrane, controlling the access of autolytic enzymes to the pneumococcal cell wall - both from within as well as from without - and/or providing a mechanism of transport of other surface-related macromolecules. We propose that the *cid* protein performs such a physiological function at some low copy number per cell, and a substantial increase in the copy number of this protein would be toxic for the bacterium (see figure 13, p. 99).

We assume that the toxicity of a high concentration of the Cid protein involves some injury (increased selective permeability?) to the plasma membrane, the integrity of which is essential for the survival of the bacteria. The same injury would

allow the release of large amounts, unregulated forms, or both of the autolytic enzymes to the cell wall. The death of completely amidase-deficient cells may be caused by the membrane injury per se, or it may be mediated by breaks (nicks) introduced into the cell wall by some wall-acting hydrolase. The latter hypothesis, however, is less convincing in view of our negative observations with the so-called Lys-2 mutants (see Appendix IV).

The model is formally analogous to the mechanism proposed for the triggering of autolytic activity by penicillin (82) and is also similar to models proposed for the mechanism of cell lysis induced by the bacteriophages lambda, ΦX174 and MS2 (22,24,30,96). An instructive precedent for such a suggestion exist in the bacteriophage lambda lytic system. While the bacterial host is lysed at the end of the virus replicative cycle, killing and lysis are clearly two separate phenomena. Bacterial death results from perforations of the plasma membrane by a so-called S protein, which channels the phage encoded lytic enzyme (lysozyme) from the cytoplasm to the periplasmic space. Lysis is due to peptidoglycan digestion by this second enzyme. Mutants in the lytic enzyme are not lysed by the phage, but extensively killed by the production of the S protein. On the other hand, mutants in the S protein are not killed by the virus and, as expected, not lysed either (see figure 13, p. 99)(96).

To envision the mechanism of autolysis-independent death, we propose that the *cid* gene is under the negative control of a repressor which is composed of cell wall material (cell wall

precursors of cell wall metabolites). We cannot speculate about the precise chemical nature of this repressor, except to suggest that its production is inhibited by antibiotics that inhibit cell wall synthesis (and maybe also DNA replication as shown by increased resistance of Cid$^-$ mutants to killing by temofloxacin, see Chapter II). We do not know whether the *cid*$^-$ mutation, which allows increased resistance to antibiotic-induced killing, is in the structural gene of the Cid protein or in some regulatory element. It is possible that the well known resistance of dormant (non-growing) bacteria to the killing effect of cell wall inhibitors is also mediated by the *cid* system.

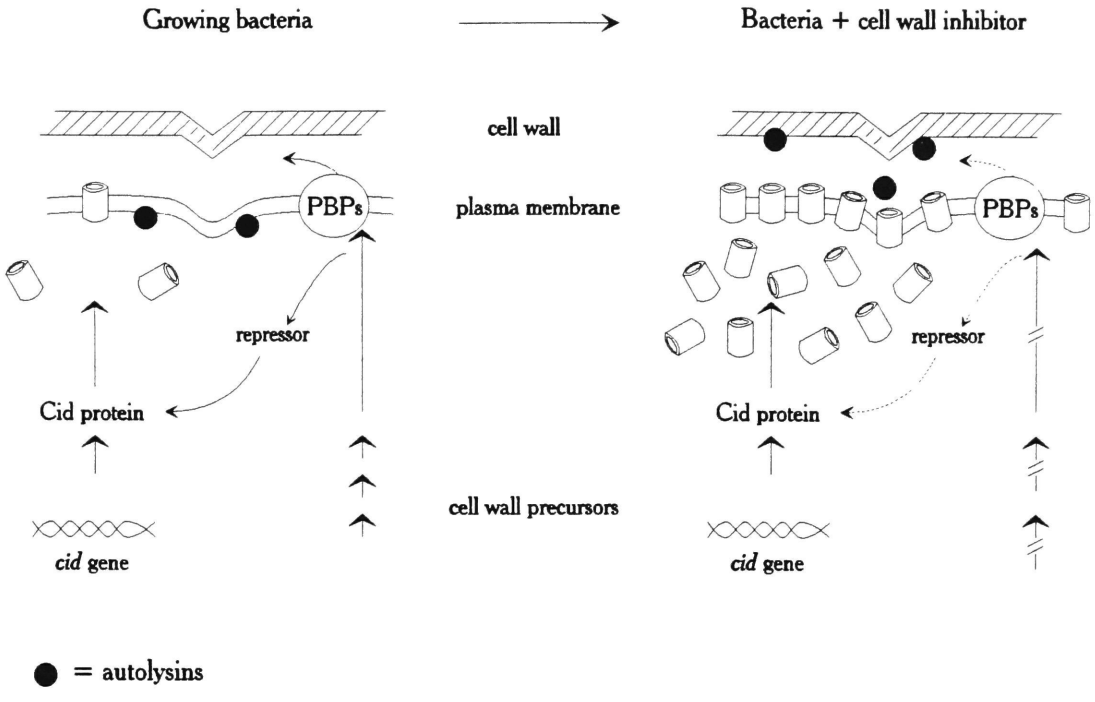

Figure 13: **Model for a mechanisms of autolysis-independent killing of pneumococci by cell wall inhibitors. Cell death is controlled by the *cid* gene product (the Cid protein), which operates at the level of the plasma membrane, controlling (directly or indirectly) the access of autolytic enzymes (black dots on the figure) to the pneumococcal cell wall and providing a mechanism of transport of other surface-related macromolecules. Cid performs such a physiological function at some low copy number per cell. During normal bacterial growth (left panel), Cid is controlled by a putative repressory circuit which is driven by the rate of cell wall (and/or other biopolymers) synthesis. Inhibition of peptidoglycan synthesis during treatment with cell wall inhibitiors (right panel) alters the Cid regulatory circuit and results in an increased production of the Cid protein. High concentrations of Cid might produce some irreversible injury to the plasma membrane and allow the release of large amounts and/or unregulated forms of autolytic enzymes to the cell wall. The death of amidase-deficient bacteria may be caused by the membrane injury per se, or may be mediated by some subtle changes (nicks) introduced in the cell wall by alternative wall-acting hydrolase. The model does not specify the number or nature of factors that participate in the killing of autolysin-defective pneumococci.**

Chapter IV

The effect of penicillin treatment on the synthesis of cellular biopolymers.

Summary of results

There is little doubt that killing and lysis of pneumococci are at the end of a pathway initiated by the inhibition of the membrane bound penicillin binding proteins by the antibiotic. While in wild-type pneumococci it is technically impossible to analyze the initial events of this pathway, the availability of isogeneic Cid^+ Lyt^- and Cid^- Lyt^- pneumococcal mutants provided an experimental system for such an analysis. The fact that neither of the mutants underwent lysis, while they differed widely in their ability to survive penicillin treatment, should permit one to differentiate between the phenomena related to cell death from phenomena that represent the largely reversible - growth inhibitory - effects of penicillin, observable in the Cid^- Lyt^- cells.

We examined in detail two specific - conceivable - mechanisms. Since it is well known that inhibition of protein synthesis renders the cells unresponsive to both penicillin-induced killing and lysis (85), was it possible that Cid^- cells were able to shutoff protein synthesis more rapidly than Cid^+ parents in response to antibiotic treatment, and thus evade the

irreversible effect of the drug treatment? A mechanism of this sort would be expected from the "unbalanced growth" model for penicillin lethality (84). The availability of Lyt$^-$ pneumococci also enabled us to test whether or not penicillin-induced killing was related to the synthesis of some toxic protein, which may be detected by a careful examination of polypeptide synthesis during penicillin treatment of Cid$^+$ and Cid$^-$ pneumococci.

The results presented in this chapter showed that the answer to the first question was negative: Cid$^-$ bacteria did not turn-off polymer synthesis faster than Cid$^+$ bacteria, indicating that this was not the mechanism preventing penicillin-induced killing in Cid$^-$ pneumococci. Examination of polypeptide synthesis in Cid$^+$ and Cid$^-$ cells yielded several unexpected, novel observations. In both types of cells, penicillin treatment resulted in a rapid and complete shutoff of protein synthesis. In addition, specific radioactive labeling of a unique protein of ca. 72 kD molecular size with [^{35}S]-cysteine was induced and/or continued, even when radioactive labeling of other bacterial proteins had come to a virtual halt. Labeling of the 72 kD protein decreased after removal of the drug from the medium leading to a resumption of bacterial growth. The 72 kD protein might be labeled in response to antibiotic-induced inhibition of biopolymer synthesis and it may participate in a regulatory circuit that shuts off polymer synthesis in penicillin-treated bacteria.

Materials and Methods

Microorganisms and growth conditions. The isogeneic pair of $Cid^+ Lyt^-$ and $Cid^- Lyt^-$ pneumococcal mutants Lyt4-4 and T6 were used in most of the experiments to be described next. In addition, some experiments were also performed with representative strains of other streptococcal species, including *S. bovis*, *S. sanguis*, *S. salivarius*, group B *Streptococcus* and *S. mitis*. All these organisms were clinical isolates susceptible to penicillin (MIC $\leq$ 0.1 ug of penicillin/ml), except for the strain of *S. mitis*, which was resistant to the drug (MIC = 2 μg/ml). Unless otherwise stated, liquid cultures of the bacteria were grown at 37°C without aeration in the chemically defined medium Cden (see Materials and Methods in Chapter I), from which the normal content of either uridine, L-phenylalanine or L-methionine had been omitted. In some experiments, this medium was supplemented with 0.1% (w/v) of yeast extract (Difco) to enhance bacterial growth. Cultures growth and viable counts were followed as described.

Antibiotics and reagents. Benzylpenicillin and the vancomycin derivative LY-A47934 were obtained from Eli Lilly and Co.; cefotaxime from Hoechst-Roussel Pharmaceuticals Inc. (Somerville, NJ); erythromycin, chloramphenicol, rifampin, mitomycin-C, streptomycin and chlorate from Sigma and penicillinase (Penicillinase Concentrate) from Becton Dickinson and Co.

(Cockeysville, MD). The radiochemicals were as follow: (i) L-[alanine-2,3-^{3}H]-phenylalanine (21.6 Ci/mmol) and [5-^{3}H]-uridine (25.8 Ci/mmol) were obtained from Dupont, NEN Research Products (Boston, MA); (ii) [^{35}S]-sodium sulphate (9.69 mCi/ml), TRAN^{35}S-LABEL (a mixture of 70% of L-[^{35}S]-methionine and 15% of L-[^{35}S]-cysteine; >1000 Ci/mmol) and L-[^{35}S]-cysteine (translation grade; >800 Ci/mmol) were obtained from ICN Biomedicals, Inc. (Costa Mesa, CA) and (iii) L-[U-^{14}C]-amino acid mixture (lacking both methionine and cysteine; 50 μCi/ml), L-[^{35}S]-methionine (>800 Ci/mmol) and L-[methyl-^{14}C]-methionine (70-85 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). The chemical used for protein electrophoresis were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals were reagent grade, commercially available products.

Drug treatment and heat shock. Bacteria were grown at 37°C in 200-500 ml cultures to an OD_{620} of 0.2-0.3 (corresponding to 0.5-1 x 10^{8} CFU). At this time, the master culture was split in 20-100 ml portions that received immediately 20x the MIC of either penicillin, or one of the other antibiotics listed above. For each drug (or temperature condition) a culture treated with penicillin was tested in parallel as a control. OD_{620} and colony counts were followed and rates of protein or RNA synthesis were determined as described below.

In certain experiments, penicillin was inactivated *in situ* after 2 h of treatment by the addition of 1,000 U of

penicillinase/ml (final concentration) to the cultures, and resumption of growth (as measured by increase in both OD_{620} and colony counts) and protein synthesis were followed. In other experiments, the effect of heat shock was investigated by transferring one portion of the culture to a 45°C water bath. Finally, in some experiments, pneumococcal (Lyt 4-4) cultures were grown in subinhibitory concentrations (1/10 - 1/2 x the MIC) of chlorate (MIC for Lyt 4-4 = 10 mM), a potent oxidant also known to inhibit protein tyrosine sulphation in eukaryotic cells (4).

Rates of protein and RNA synthesis. Strains of pneumococci were grown at 37°C in 100 ml volumes of Cden minus phenylalanine and uracil, but supplemented with yeast extract. At an OD_{620} of 0.2 to 0.3 (corresponding to 0.5-1 x 10^8 CFU/ml), 20x the MIC of penicillin was added to the cultures and incubation was continued. Rates of synthesis of protein and RNA were determined by pulse-labeling the cells with [^{3}H]-phenylalanine or [^{3}H]-uridine at several times before and after the addition of penicillin: 0.950 ml samples of the cultures were pipetted into prewarmed (37°C) borosilicate tubes (12 x 75 mm) containing 50 μl of either [^{3}H]-phenylalanine (5 μCi and 0.04 ug) or [^{3}H]-uridine (5 μCi and 0.044 ug), incubated for 5 min at 37°C, and rapidly frozen in dry ice. Eventually, the samples were thawed, treated for 30 min with 10% (w/v; final concentration) ice cold trichloroacetic acid to precipitate proteins and RNA, and

filtered through either 0.45 μm pore size filters (Millipore) (for proteins) or glass microfiber filters (Whatman, Inc., Clifton, NJ) (for RNA). The filters were rinsed extensively with a 10 mg/100 ml solution of cold phenylalanine or uridine, dried, and transferred to vials containing Ready Safe liquid scintillation cocktail (Beckman Instruments). All [^{3}H]-labeling experiments were performed in triplicate.

Radiolabeling of proteins for gel electrophoretic analysis. At several time points before and during drug or heat treatment, the cultures were labeled with various radioactive precursors. Aliquots (4.5 ml) of the cultures were transferred into prewarmed (37°C) 15 ml plastic-capped tubes containing adequate amounts of radioactive tracer (see details below) diluted in 0.5 ml of medium. Incubation was continued for 7 min before labeling was interrupted by one of the methods to be described next.

(i) For pulse labeling experiments, labeling was stopped by the addition of 5 ml of ice cold 20 mM Tris HCl pH 8 buffer containing a 50-100 x excess of the same non-radioactive precursor, in order to dilute out the radioactive tracer. The radioactively labeled cells were harvested by centrifugation (at 4°C), thoroughly washed 2x with 10 ml of the same buffer and resuspended in a final volume of 50-100 μl of buffer. The cells were lysed either enzymatically (by addition of 0.1% of DOC and ca. 20 U of purified amidase followed by incubation for 15 min at 37°C) or by sonication for 7-10 min in an Ultrasonic Cleaner

(Branson Cleaning Equipment Company, Shelton, CT). Successful lysis (i.e., lysis of >99% of the cells) was assessed by phase contrast microscopy.

(ii) For pulse-chase experiments, the cells were submitted to a similar 7 min radioactive pulse, but were then filtered through a 0.45 μm HA Millipore filter (Millipore Corp., Bedford, MA) saturated with "cold" precursor, and copiously washed with plain prewarmed medium before being resuspended at an OD_{620} of 0.2-0.3 in plain medium. Incubation was continued for a determined period of time before the reaction was stopped and the samples were processed and lysed as described above.

(iii) Finally, in some experiments, protein synthesis of the bacteria was blocked prior to radiolabeling by filtration-resuspension of the cells in Cden lacking the essential amino acid leucine.

Radiolabeled precursors and specific activities. The radioactive tracers used were as follows: (i) a mixture of L-[^{35}S]-methionine (40 μCi/ml and 500 nmol/ml) and L-[^{35}S]-cysteine (8,5 μCi/ml and 200 nmol/ml), (ii) L-[^{35}S]-methionine (20 μCi/ml and 500 nmol/ml), (iii) L-[^{35}S]-cysteine (2 μCi/ml and 200 nmol/ml), (iv) [^{35}S]-sulphate (sodium salt; 2.5 μCi/ml and 0.1 nmol/ml), (v) L-[methyl-^{14}C]-methionine (1.3 μCi/ml and 500 nmol/ml) and (vi) L-[U-^{14}C]-amino acid mixture (lacking both methionine and cysteine; 0.5 μCi/ml and approximately 100 μg/ml).

Protein analysis. The protein profile of the cell lysates was analyzed on one and two dimensional polyacrylamide gel electrophoresis (PAGE), in reducing conditions (46,66). Isoelectrofocusing (IEF) was run on 8% PAGE containing 8 M of urea, 2% (v/v) of NP40 and 5% (w/v) of a mixture of 1/3 of ampholines of pH 3-10 and 2/3 of ampholines of pH 4/6 (Bio-Lytes). SDS-PAGE was run on 10% PAG containing 0.1% (w/v) of SDS.

Peptide mapping of selected protein bands was performed by partial protein digestion with V8 protease (Sigma). In brief, gel slices containing the band of interest were cut out of the first 10% SDS-PAGE and, after thorough washing with 0.125 M Tris buffer at pH 6.8, loaded sideways on a second (15%) SDS-PAGE which was surmounted by a stacking gel containing 0.2 ug of V8 protease/ml and devoid of SDS.

The gels were stained with Coomassie blue or silver stain and dried, either directly or after soaking in Enlightning (NEN), before exposure to Kodak X-OMAT AR films (Eastman Kodak Company, Rochester, NY) for autoradiography or fluorography. Intensities of the radioactive bands were quantified by autoradiography on storage phosphor screens, using the PhosphorImager and the ImageQuant software version 3.0 (Molecular Dynamics, Sunnyvale, CA).

Amino acid analysis. In certain experiments, it was necessary to determine the presence of L-[^{35}S]-cysteine in the radiolabeled

proteins. Total (radiolabeled) cell proteins were precipitated with 10% ice cold trichloroacetic acid (for 30 min), thoroughly washed with acetone, dried and oxidized with performic acid to convert cysteine to cysteic acid in order to protect its sulphur group from hydrolysis during further acid hydrolysis of the peptide bonds. Amino acids were hydrolyzed for 20 h at 110°C in 6 N HCl and separated on a Dowex (anion exchange) column (kindly performed by Dr. Manning, The Rockefeller University). Amino acid fractions were recovered and tested for the presence of radioactivity by liquid scintillation counting. Separation of [^{35}S]-cysteic acid from possible contamination with [^{35}S]-sulphate was performed by paper chromatography using a solvent containing 10 ml of ethanol, 4.5 ml of H_2O and 0.5 ml of acetic acid.

For amino acid sequencing of radiolabeled protein peptides, the protein band of interest was partially digested with V8 protease followed by separation of the peptides by SDS-PAGE (as described for peptide mapping, see above). The peptides were transferred to Immobilon-P (PVDF) Transfer Membrane (Millipore) using the MilliBlot-SDE Transfer System (Millipore). After autoradiography of the membranes, the desired radioactive spots were cut out and pooled for further amino acid sequencing. Protein analysis was provided by the Rockefeller University protein sequencing facility.[9]

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[9]Supported in part by NIH shared instrumentation grants and by funds provided by the U.S. Army and Navy for purchase of equipment.

Results

Synthesis of biopolymer in pneumococci treated with penicillin.

I. Kinetics of growth inhibition after the addition of penicillin. We used isogeneic pairs of Cid^- Lyt^- and Cid^+ Lyt^- cultures to compare the rates with which Cid^- and Cid^+ cells slowed growth rates (as measured by increase in OD_{620}) and rates of protein and RNA synthesis after the addition of penicillin. Figure 14 (p. 110) shows that the rates of growth and protein and RNA synthesis decreased in the two cultures at virtually identical initial rates. The differences in the rates of protein and RNA synthesis after longer times of incubation with penicillin presumably reflected the very substantial difference in the number of viable bacteria in the Cid^- versus those in the Cid^+ cultures (figure 14-A). However, the decrease in the rates of macromolecule synthesis over time was not proportional to loss of viability. At 1 h, for instance, the rates of protein synthesis were still virtually identical in both cultures, whereas Cid^+ cells had already lost $\geq$10x more viable cells than Cid^- cultures. A similar trend was also observed for RNA synthesis.

Cid^+ and Cid^- cells also synthesized similar amounts of residual cell walls after addition of penicillin. This was shown by equal incorporation of radioactivity/mg of purified cell walls in bacteria labeled for 2 h with [^{3}H]-choline after the addition

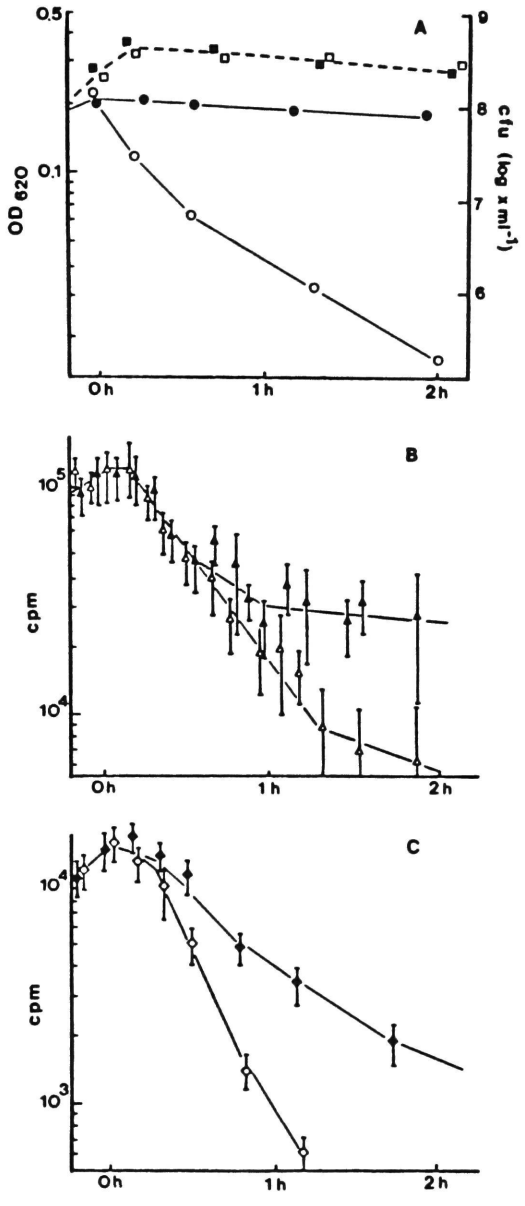


Figure 14: Kinetics of growth inhibition and loss of viability after the addition of penicillin. (A) Cultures of isogeneic pairs of Cid^+ Lyt^- (Lyt 4-4; open symbols) and Cid^- Lyt^- (T6; closed symbols) pneumococci in the exponential phase of growth received 20x the MIC of penicillin at 0 h, and the effect of antibiotic treatment on the cultures' OD_{620} (dashed lines) and loss of viability (solid lines) was monitored. Panels B and C show the effect of penicillin treatment on the rates of proteins (B) and RNA (C) synthesis, as determined by pulse-labeling samples of the cultures for 5 min with either [^{3}H]-phenylalanine (panel B) or [^{3}H]-uridine (panel C), followed by measurement of the number of counts per minute incorporated by the cells in trichloroacetic acid-precipitable macromolecules during this period of time (see text). Each dot in panels B and C represent the mean $\pm$ standard deviation of three determinations.

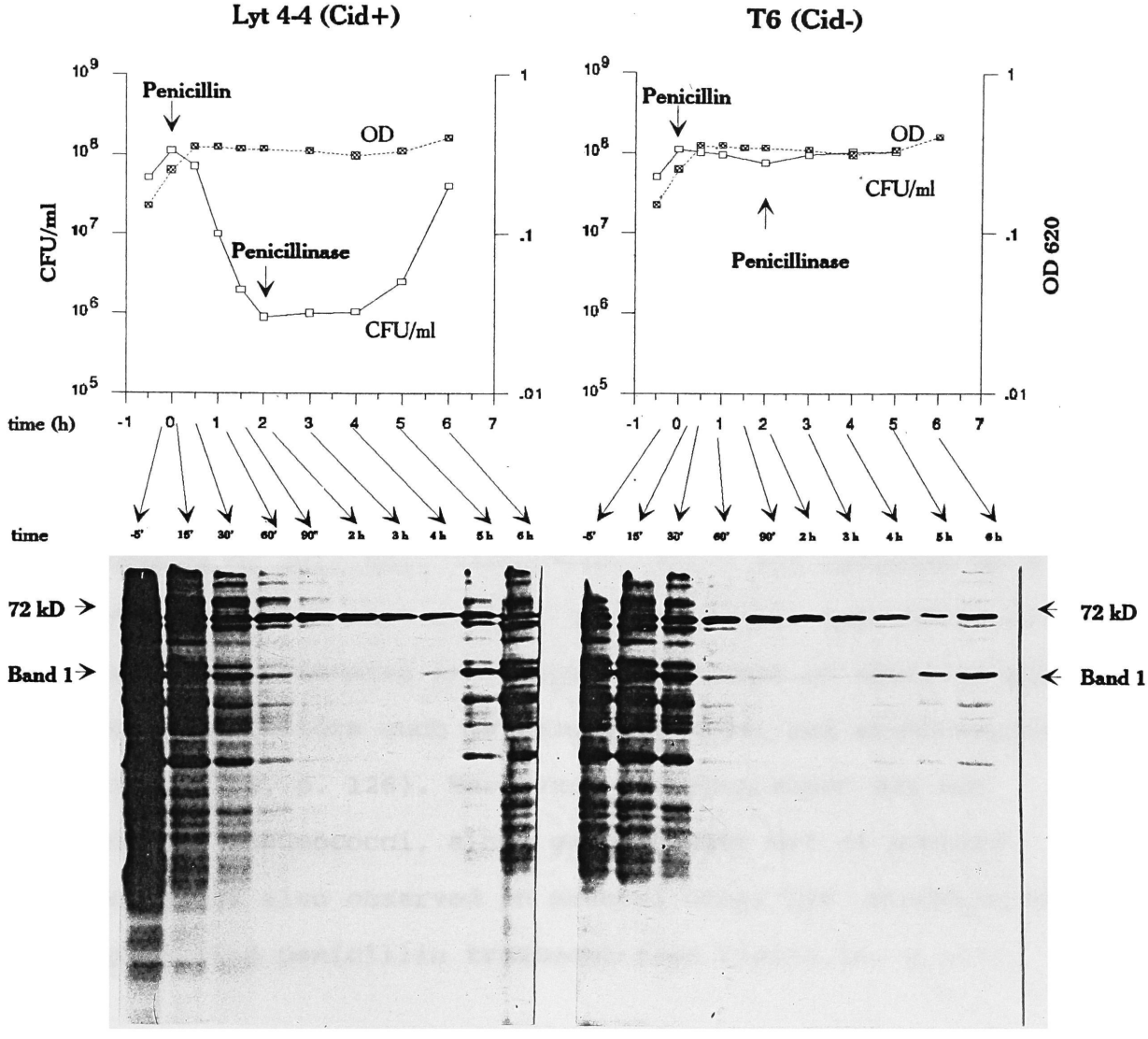
of the drug (i.e., 6x 10^4 cpm/mg of wall for Cid^+ cells and 7x 10^4 cpm/mg for Cid^- bacteria).

Thus, no major differences were detectable in the initial rates of growth inhibition in these two organism, and inhibition of polymer synthesis occurred with the same initial rates in both cases. The next series of experiments present a more detailed analysis of the shutoff of protein synthesis produced by treatment with penicillin.

II. The effect of penicillin on protein synthesis in pneumococci. Cultures of Cid^+ Lyt^- and Cid^- Lyt^- pneumococci were pulse-labeled with a mixture of [^{35}S]-methionine and [^{35}S]-cysteine (Tran^{35}Slabel) at various times before and after the addition of 20x the MIC of penicillin to the bacteria. Incorporation of radioactive amino acids in proteins was quantified by autoradiography using the PhosphorImager after separation of the polypeptides by SDS-PAGE. Figure 15 A and B (p. 112) show that in both kinds of pneumococcal cultures incorporation of radioactive amino acids underwent a rapid decline following administration of the antibiotic, which confirmed the phenomenon observed in the previous section (figure 14, p. 110). However, another striking feature of the autoradiogram, besides the complete halt of synthesis of most polypeptides, was the continued (and/or induced) labeling of a single band of approximately 72 kD molecular size.

In the following, we shall briefly analyze first the shutoff

Figure 15: **Shutoff of protein synthesis during penicillin treatment. Cultures of the Cid^+ Lyt^- strain Lyt 4-4 (left panel) or the Cid^- Lyt^- strain T6 (right panel) were treated in the exponential phase of growth with 20x the MIC of penicillin. After 2h of treatment, penicillin was inactivated by the addition pencillinase (10^3 U/ml) to the medium. At several time-points before and after addition of penicillin, samples were removed and pulse-labeled for 7 min with "Tran^{35}Slabel" before being lysed and processed for separation by SDS-PAGE and autoradiography. The upper part of the figure shows the OD_{620} (closed symbols) and viable counts (open symbols) during the course of the experiment. The lower part of the figure shows the autoradiograms of the radiolabeled cellular proteins. The lanes were loaded with equal amounts of proteins. No differences in the protein profiles were observed on Coomassie blue stained gels.**


Lyt 4-4 (Cid+)
T6 (Cid-)
Penicillin
OD
Penicillinase
CFU/ml
CFU/ml
OD 620
time (h)
time
-5'
15'
30'
60'
90'
2 h
3 h
4 h
5 h
6 h
72 kD
Band 1
72 kD
Band 1

of protein synthesis, and then the nature and regulation of the 72 kD radiolabeled species.

II.1 Shutoff of protein synthesis. Figure 15 A and B show that incorporation of the tracer had come to a virtual halt in all the polypeptides (except the 72 kD species) after about 90-120 min of exposure to penicillin. During this period of time, no striking changes had occurred in the profile of the bacterial bulk proteins as visualized on Coomassie blue stained gels. Both Cid^+ and Cid^- cultures stopped growing (as measured by the OD_{620}), however, Cid^+ cells lost viability (about 2 Log units in 2 h) while there was only a minor decline in viable titers in the Cid^- culture ($\leq$ 0.5 Log unit in 2 h). The disproportionality between loss of viability and decline in protein synthesis was even more striking in figure 16 (p. 114), which shows the quantitation of the radioactive labeling of the protein bands by densitometry on the PhosphorImager (Molecular Dynamics). It can be seen that while survival of Cid^+ and Cid^- cells was very different (open squares in the figure), the reduction in the rates of synthesis of most polypeptides (as measure by the total intensity of the lanes on the autoradiograms) and that of a major band "I" (for its remarkable decrease in intensity during penicillin treatment) were almost identical. Moreover, after *in situ* inactivation of penicillin by penicillinase, protein synthesis resumed somewhat faster than cell division (as measured by the increase in colony counts) in both kinds of organisms.

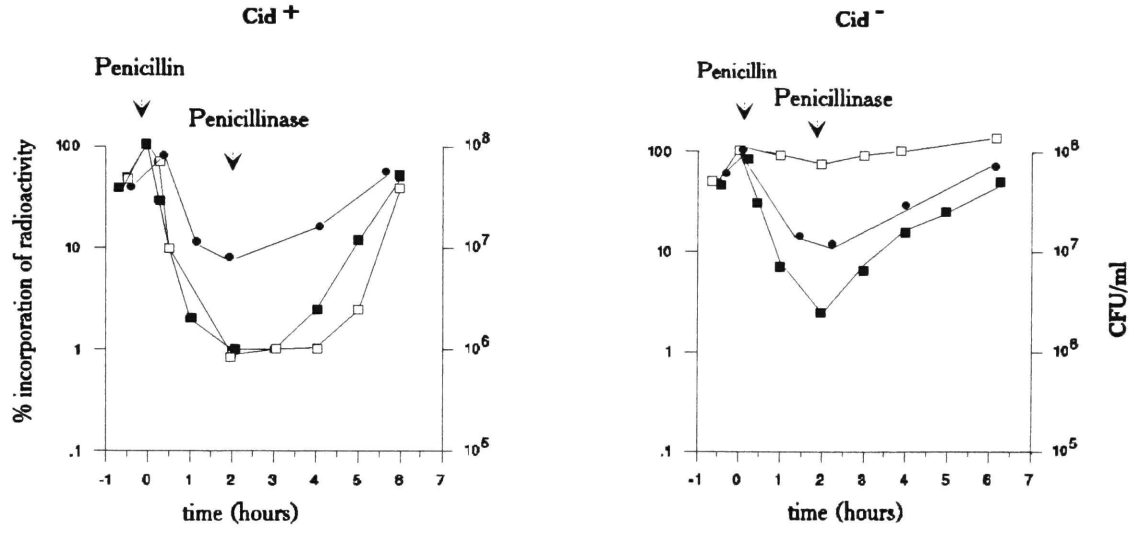


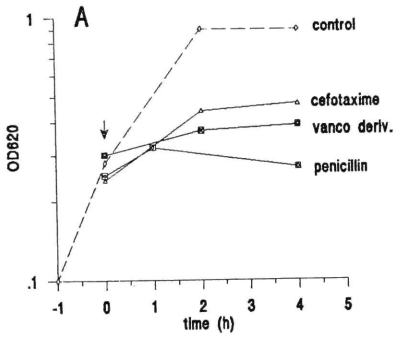
Figure 16: Kinetics of penicillin-induced killing (open symbols) and shutoff of protein synthesis (closed symbols) were measured in cultures of Cid^+ Lyt^- (strain Lyt 4-4; left panel) and Cid^- Lyt^- (strain T6; right panel) pneumococci. Cultures of either strains were treated with 20x the MIC of penicillin and pulse-labeled with "Tran^{35}Slabel" and the protein content of cell lysates were analyzed by SDS-PAGE and autoradiography as in figure 16. Intensities of the bands were quantified using the PhosphorImager and the ImageQuant software version 3.0 (Molecular Dynamics). Shutoff of total protein synthesis (closed circles) was determined by measuring the intensities of total lanes. Shutoff of so-called Band 1 (closed squares; see figure 16), was followed for comparison because it represented a major protein species on the gel.

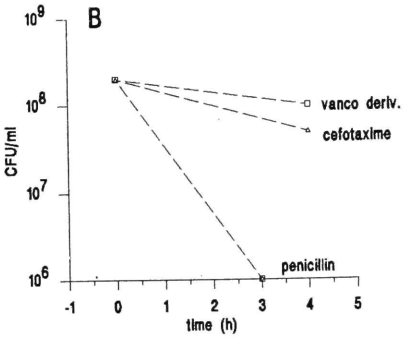
This observation suggested the possible existence of a global regulatory mechanisms, which was triggered by penicillin treatment and resulted in shutoff of protein synthesis, whether or not the cells had been killed (i.e., were unable to divide) by the drug. This hypothesis was confirmed by experiments in which Cid^+ Lyt^- pneumococci were treated with non-cidal cell wall inhibitors. Figure 17 (p. 116) shows that the ß-lactam cefotaxime and the vancomycin derivative LY-A47934 resulted in a substantial inhibition of protein synthesis (and also in the labeling of the 72 kD band), in spite of the fact that they did not kill Lyt 4-4 pneumococci.

Thus, inhibition of protein synthesis was a genuine consequence of cell wall inhibition, which was mediated by a regulatory circuit that has yet to be defined. Inhibition of synthesis was extensive as it approached that of specific protein synthesis inhibitors such as chloramphenicol and erythromycin (see figure 22, p. 126). Moreover, the phenomenon was not confined to pneumococci, since general shut off of protein synthesis was also observed in several other Lyt^- streptococcal species during penicillin treatment (see figure 18, p 117).

II.1 The nature and regulation of the 72 kD radioactively labeled band. Penicillin treatment of wild-type pneumococci is known to cause massive perturbation of the cells surface. While autolysis and viability loss were eliminated in the particular

Figure 17: **Cultures of the Cid^+ Lyt^- strain Lyt 4-4 in the exponential phase of growth were treated with 20x the MIC of either penicillin, cefotaxime or the vancomycin derivative LY-A47394 at 0 h (arrow). OD_{620} (panel A) and viable counts (panel B) in the cultures were followed. At different time-points, samples of the cultures were removed, pulse-labeled with "Tran^{35}Slabel" as described, lysed and processed for autoradiography. The autoradiograms are shown at the bottom of the figure. Ctx stands for cefotaxime and V for the vancomycin derivative LY-A47394.**


A
1
.1
OD620
control
cefotaxime
vanco deriv.
penicillin
-1
0
1
2
3
4
5
time (h)


B
10^9
10^8
10^7
10^6
CFU/ml
vanco deriv.
cefotaxime
penicillin
-1
0
1
2
3
4
5
time (h)

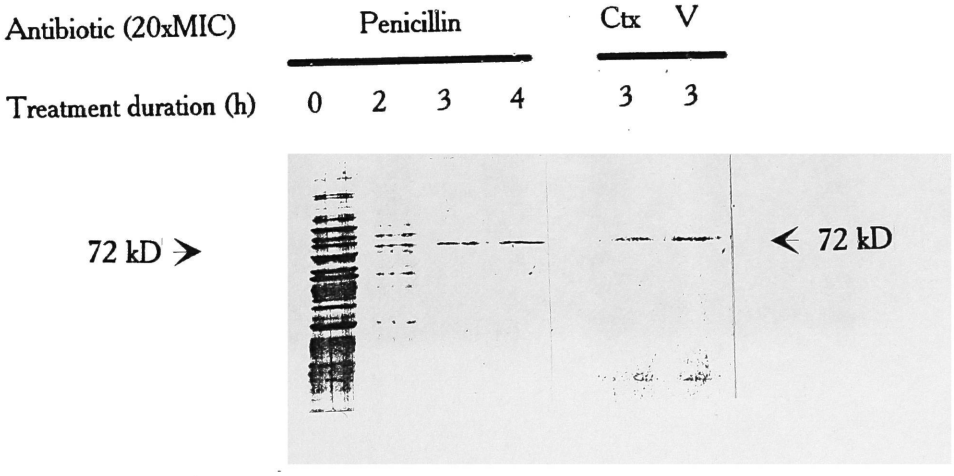
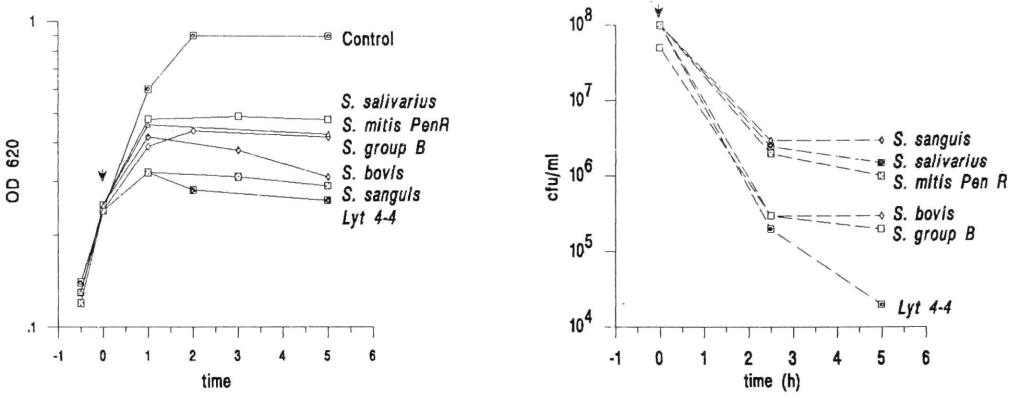
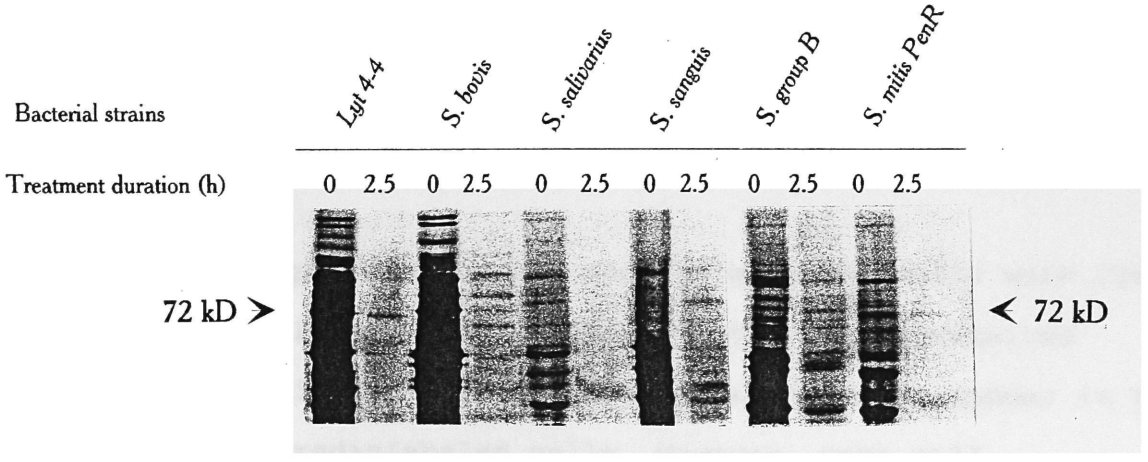

Antibiotic (20xMIC)
Penicillin
Ctx
V
Treatment duration (h)
0
2
3
4
3
3
72 kD
72 kD

Figure 18: **Penicillin-induced shutoff of protein synthesis was also observed in non-pneumococcalstreptococci. Cultures of clinical isolates of bacteria representative of several different species of streptococci were treated with 20x the MIC of penicillin (arrow in panel A) and OD620 and viable counts in the cultures were followed (panel A). At different time-points, samples of the cultures were removed, pulse-labeled with "Tran^{35}Slabel" as described and processed for autoradiography. Panel B shows the autoradiograms of samples labeled just prior to the addition of penicillin to the cultures (0 h) and 2. 5 h later. For each bacterial species, the lanes were loaded with similar amounts of proteins.**

A


OD 620
time
Control
S. salivarius
S. mitis PenR
S. group B
S. bovis
S. sanguis
Lyt 4-4
cfu/ml
time (h)
S. sanguis
S. salivarius
S. mitis Pen R
S. bovis
S. group B
Lyt 4-4

B


Bacterial strains
Lyt 4-4
S. bovis
S. salivarius
S. sanguis
S. group B
S. mitis PenR
Treatment duration (h)
0 2.5 0 2.5 0 2.5 0 2.5 0 2.5 0 2.5
72 kD
72 kD

mutants used on our experiments, production of more complex artifacts during the penicillin treatment of the latter mutants could not be *a priori* excluded. For this reason, we performed a series of experiments aimed at the elucidation of the nature of the radioactively labeled 72 kD band.

(i) The 72 kD band is a protein. The 72 kD band was completely destroyed by digestion with trypsin, but not by trypsin plus soybean protease inhibitor. The band was also digested by V8 staphylococcal protease and pronase, but not by nucleases.

Conditions of partial proteolysis with V8 protease resulted in the production of several radioactively labeled peptides (figure 19, p. 119). The figure also shows that under the same conditions of partial proteolysis a 72 kD radioactively labeled material produced by pneumococci treated with chloramphenicol (instead of penicillin) produced a set of radioactively labeled peptides which were indistinguishable from the proteolytic products produced from the protein seen in penicillin-treated cells.

The 72 kD protein did not show up when the cells were lysed prior to pulse-labeling, indicating that labeling required cellular integrity. The 72 kD labeled band did not appear in the supernatant of radiolabeled cells. However, upon cell fractionation, the band partitioned in approximately equal amounts in both soluble and particulate (membrane enriched) fractions. The band was apparently not attached to the bacterial

Figure 19: **Peptide mapping of the 72 kD radiolabeled protein. Cultures of Lyt 4-4 were treated with 20x the MIC of penicillin (or chloramphenicol), pulse-labeled as described and proteins of cell lysates were separated by SDS-PAGE (panel A). For partial proteolytic mapping, the lanes containing the 72 kD protein were cut out and loaded sideways on a second SDS-PAG on top of which the stacking gel was devoid of SDS and contained V8 protease (panel B; see text for details). The peptides were separated by electrophoresis and the second dimension gel was processed for autoradiography. Radioactive peptides are indicate by the arrow-heads in panel B.**

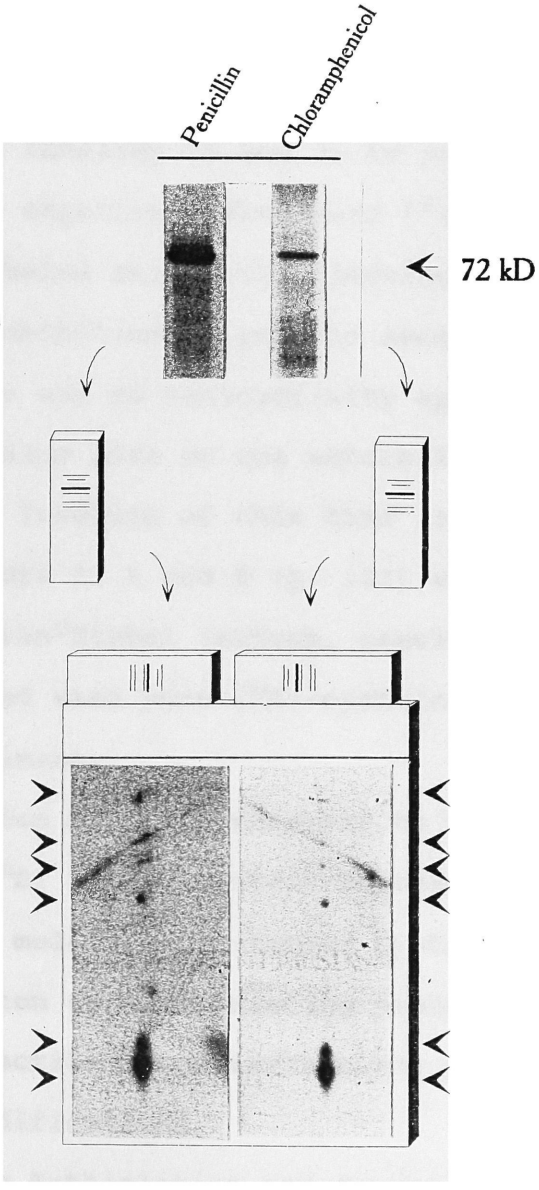
A.

Cut lane and load sideways on V8-protease containing gel.

B.

V8 protease containing stacking gel

15% SDS- PAGE



cell walls, because walls had been enzymatically digested by amidase during cell lysis. Therefore, the protein was likely to be partly attached to the plasma membrane. Interestingly, treatment by high salt (1M NaCl or 1M LiCl) did not release the band from the particulate fraction (see figure 32, appendix VIII, p. 155)

(ii) Nature of the labeling of the 72 kD protein. Upon repeating the pulse-labeling experiment with pure [^{35}S]-methionine or with mixture of [^{14}C]-labeled amino acid (lacking cysteine) it was found that while inhibition of protein synthesis with penicillin was complete, there was no radioactivity appearing in the region of the 72 kD molecular size on the autoradiograms. This finding suggested that the labeling of this band in the experiments illustrated in figure 15 A and B (p. 112) was due to the second component of the Tran^{35}Slabel isotope, namely [^{35}S]-cysteine. This was indeed confirmed when pure [^{35}S]-cysteine was used as a tracer in the basic experiment.

This observation made it necessary to test whether incorporation of [^{35}S] in the protein represented incorporation of the total cysteine molecule or whether cysteine merely acted as a donor of sulphur atom to a preexisting protein. The results of the tests to be described next exclude the most common sulphur-related protein modifications.

First, protein S-thiolation and formation of thio-ester bonds were unlikely because the radioactive label of the 72 kD

protein was retained during (i) boiling for 5 min in 0.1% of SDS plus 10 to 50 mM of either dithiothreitol or dimercaptoethanol (which reduces S-S bonds) and (ii) exposure for 18 h to 0.5 M of hydroxylamine at pH 7 (which breaks thio-ester bonds). Second, protein tyrosine sulphation, a common protein modification in eukaryotic cells, but not in bacteria (38), was unlikely because (i) the 72 kD protein could not be labeled by Na_2-[^{35}S]-O_4 (4) and (ii) there was no loss of label after exposure to 1 M HCl for 1 h at room temperature plus 5 min at 95°C, a treatment which breaks S-O-tyrosine bonds (37). Moreover, pretreatment of the cells with the oxidant chlorate (a potent inhibitor of tyrosine sulphation in eukaryotic cells (4)) did not interfere with *in vivo* labeling of the band. Finally, cellular proteins radiolabeled in the 72 kD band were oxidized with performic acid followed by hydrolysis in 6N HCl overnight and amino acid analysis. All the radioactivity was recovered with the material eluting at cysteic acid and/or inorganic sulphate. The radioactive material recovered from the amino acid analysis was then separated by paper chromatography in a solvent capable of separating cysteic acid from sulfuric acid. The radioactivity was recovered with the cysteic acid fraction and was apparently not due to contamination with inorganic sulphate.

Thus, the most common forms of protein modification, such as S-thiolation (72), formation of thio-ester or thio-ether (such as lanthionine, which results from the condensation of two cysteines: COOH-CNH2-CH2-S-CH2-CNH2-COOH) and protein sulphation

seemed to be excluded. Therefore, labeling of the 72 kD protein probably resulted from (non-ribosomal?) addition of whole cysteine residues to the protein, possibly via peptide bonds.

(iii) Regulation of the 72 kD protein labeling. The intensity of labeling of the 72 kD band appeared to obey some regulatory mechanism(s) of the cells. Figure 20 (p. 123) shows that after addition of penicillin, non-growing bacteria labeled the 72 kD protein for several hours at practically the same rate, as determined by pulse-labeling experiments. In contrast, however, as soon as penicillin was inactivated *in situ* (by addition of penicillinase to the cultures), labeling of the 72 kD band sharply decreased, while both synthesis of bulk proteins and cell division resumed. The decreased labeling of the 72 kD band after resumption of bacterial growth was not due to a lack of intracellular tracer, as shown by the fact that radioactive amino acids were incorporated in all other polypeptides.

This observation was confirmed by 2-D gel electrophoresis, a technique which allowed a better resolution (figure 21, p. 124). However, it was not possible to determine whether some labeling of the band also occurred at some very low percentage of total radioactive incorporation in normally growing bacteria, because the area of the 72 kD species was overshadowed by labeling of other proteins. Labeling of the 72 kD protein also decreased when penicillin treated cells were starved of the essential amino acid leucine, a condition which inhibits protein synthesis in non-

Figure 20: **The labeling of the 72 kD protein decreased after inactivation of penicillin with penicillinase, while synthesis of the other protein species resumed. A culture of the Lyt⁻ pneumococcal mutant Lyt 4-4 was treated with 20x the MIC of penicillin and samples were pulse-labeled (for 7 min) with "Tran^{35}Slabel" at various time-points of the treatment as described. After 2 h, one portion of the culture received penicillinase (10^3 U/ml) to inactivate penicillin, while the other portion continued to be exposed to the antibiotic. The upper part of the figure shows the autoradiograms of SDS-PAGEs from lysates of pulse-labeled cells. The lower part of the graph shows the intensities of the 72 kD band and of so-called "Band 1" (for comparison) as determined by the "PhosphorImager". Band intensities are expressed in arbitrary units. The lanes were loaded with equal amounts of total proteins.**

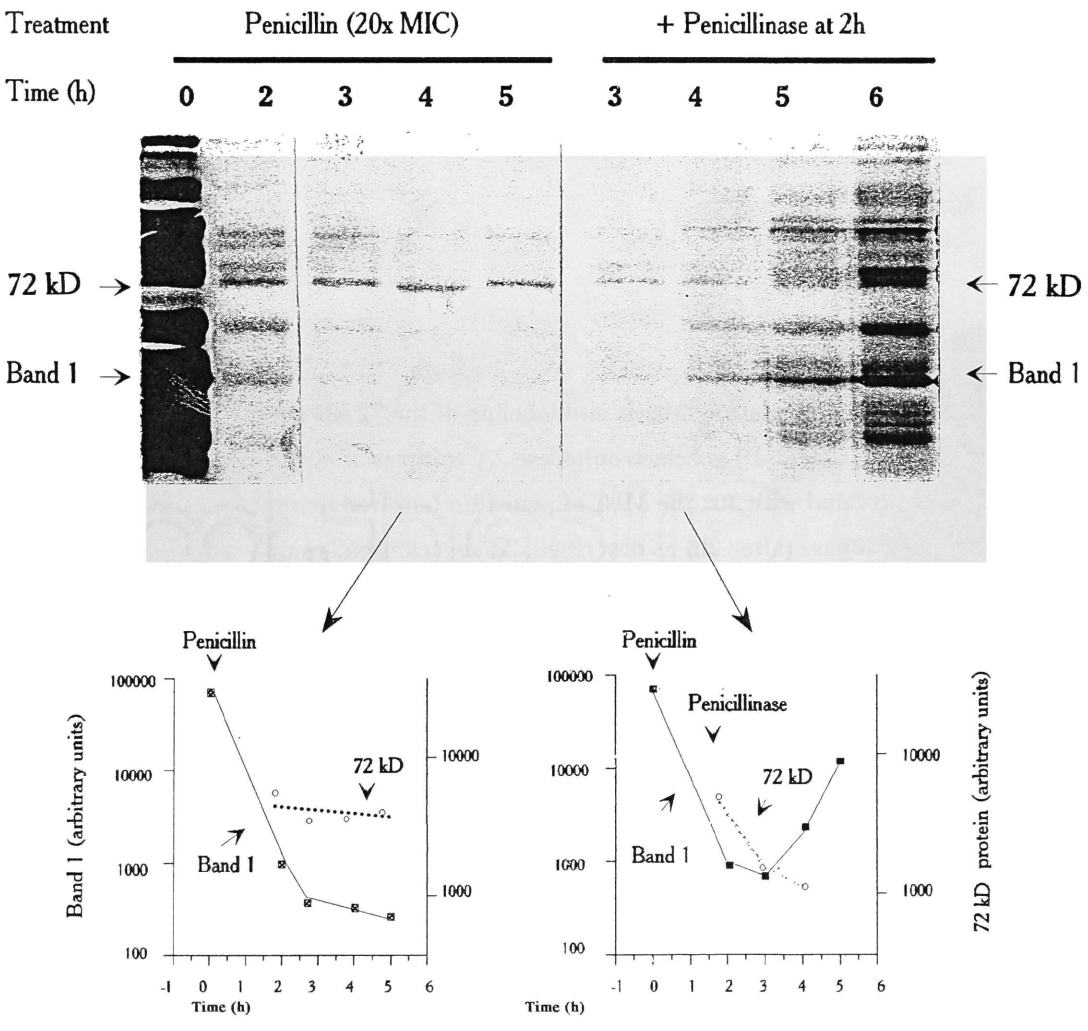
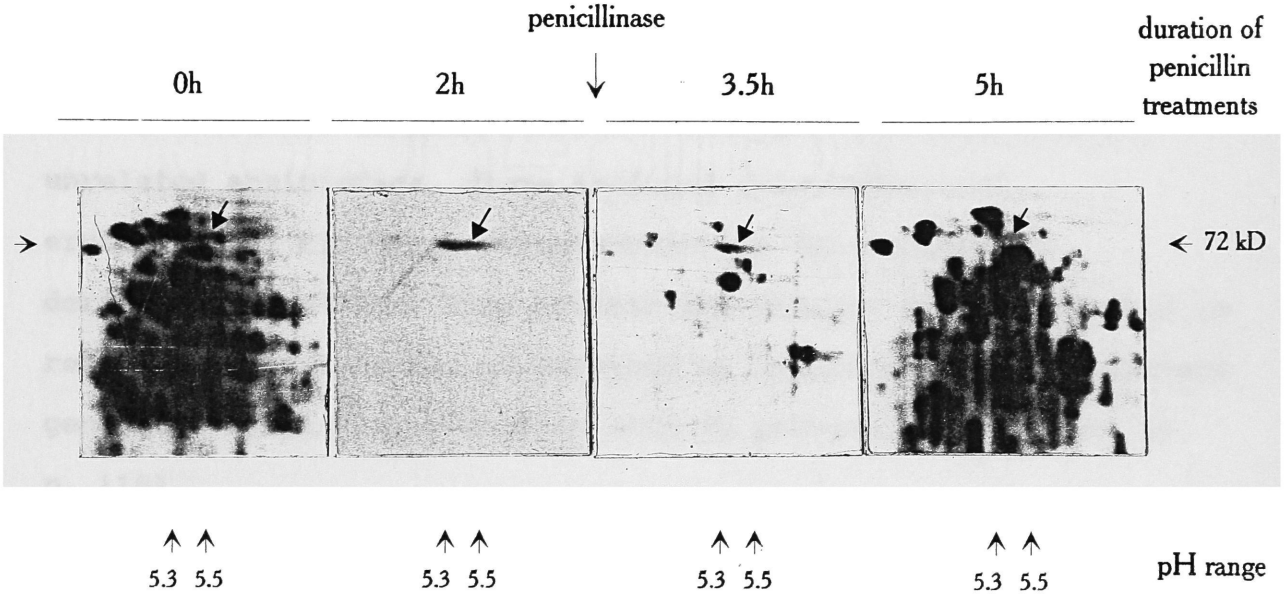

Treatment
Penicillin (20x MIC)
+ Penicillinase at 2h
Time (h)
0
2
3
4
5
3
4
5
6
72 kD
Band 1
72 kD
Band 1
Penicillin
Band 1 (arbitrary units)
100000
10000
1000
100
72 kD
Band 1
10000
1000
-1
0
1
2
3
4
5
6
Time (h)
Penicillin
Penicillinase
72 kD
Band 1
72 kD protein (arbitrary units)

Figure 21: **Shutoff of protein synthesis and labeling of the 72 kD species depicted by 2-dimensional (2-D) gel electrophoresis. A cultures of strain Lyt 4-4 (Cid^+ Lyt^-) was treated with 20x the MIC of penicillin followed by inactivation by penicillinase (after 2h) as described. At several time-points, samples of the cultures were pulse-labeled with "Tran^{35}Slabel" and processed for 2-D gel electrophoresis and autoradiography. After inactivation of penicillin with penicillinase, global protein synthesis resumed, while labeling of the 72 kD species decreased. The position of the 72 kD radiolabeled protein is indicated by the arrows. Similar amounts of total proteins were loaded on the gels.**


penicillinase
duration of penicillin treatments
0h
2h
3.5h
5h
72 kD
5.3 5.5
5.3 5.5
5.3 5.5
5.3 5.5
pH range

treated pneumococci (data not presented).

Finally, pulse-chase experiments showed that (i) bulk proteins radiolabeled prior to penicillin treatment remained unchanged for up to 4 hours (the duration of follow up) of penicillin treatment and (ii), the radiolabeled 72 kD protein was also stable for up to 2 h (the duration of follow up) of exposure to the antibiotic.

(iv) Labeling of the 72 kD protein in other conditions.
Figure 22 (p. 126) shows that the 72 kD protein was also labeled during treatment of pneumococci with other, mechanistically unrelated antibiotics. These included chloramphenicol, erythromycin, rifampin, streptomycin and the vancomycin derivative LY-A47934. This protein was similar to that labeled in response to penicillin, as assessed by identical peptide patterns generated by partial digestion with V8 protease (see figure 19, p. 119).

In contrast, however, the DNA cross-linking agent mitomycin resulted in very poor labeling (if at all) of the protein (figure 22). In addition, the 72 kD protein was not specifically labeled during heat shock (45°C for 2 h), while two other major protein species (molecular weight about 50 and 30 kD resp., see figure 23, p. 127) were still being produced in response to this treatment. Thus, the ability to label the 72 kD protein was shared by a number of stress conditions, but most interestingly not by heat shock.

Figure 22: **Global shut-off of protein synthesis and labeling of the 72 kD band during treatment of the Lyt$^-$ pneumococcal mutant Lyt 4-4 with 20x the MIC of various antibiotics mechanistically unrelated to penicillin (except for the β-lactamine cefotaxime). The cells were pulse-labeled at various times with "Tran^{35}Slabel", lysed and separated by SDS-PAGE before autoradiography as described. Lanes were loaded with equal amounts total proteins. V = vancomycin derivative, Ctx = cefotaxime and Str = streptomycin.**

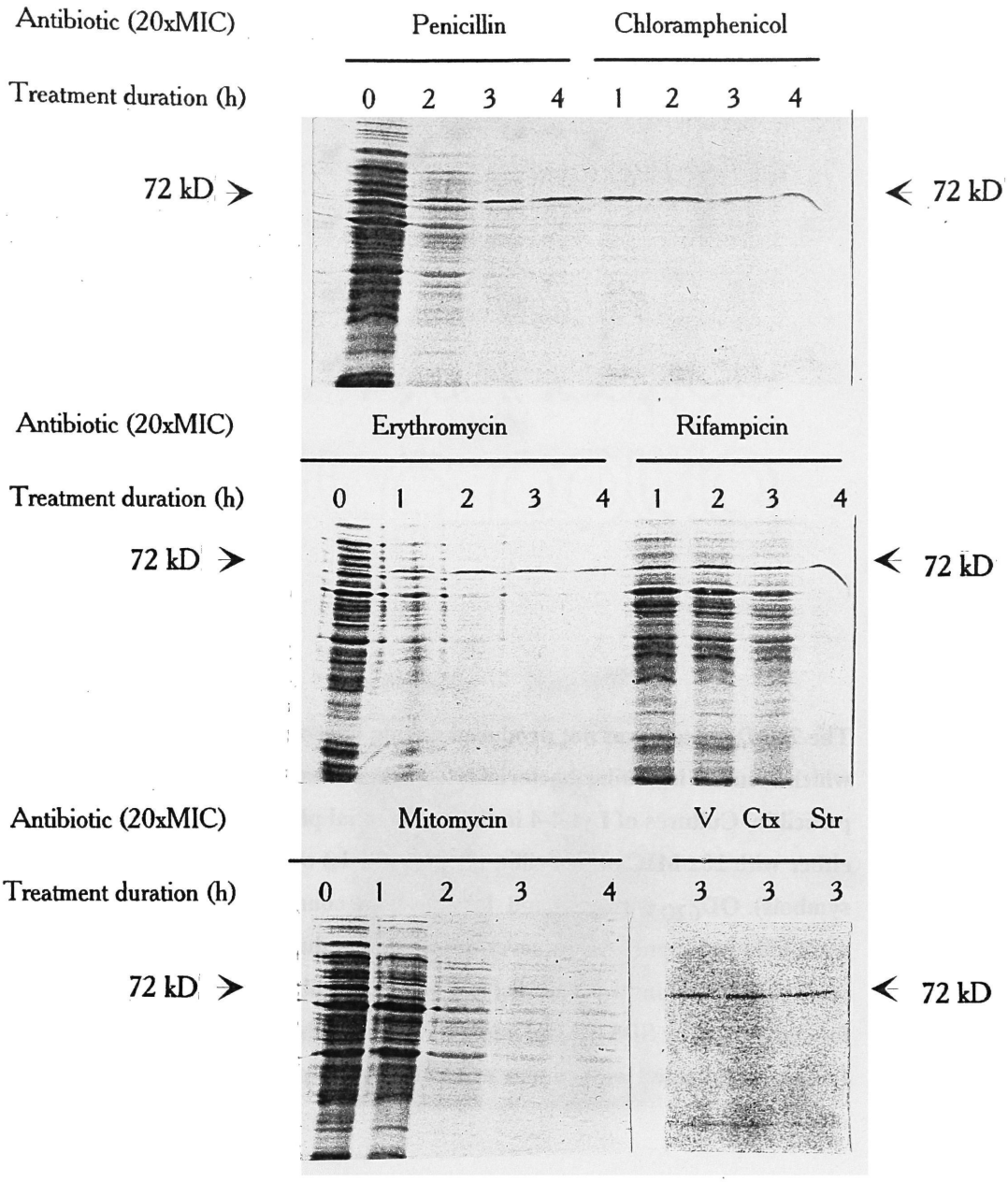
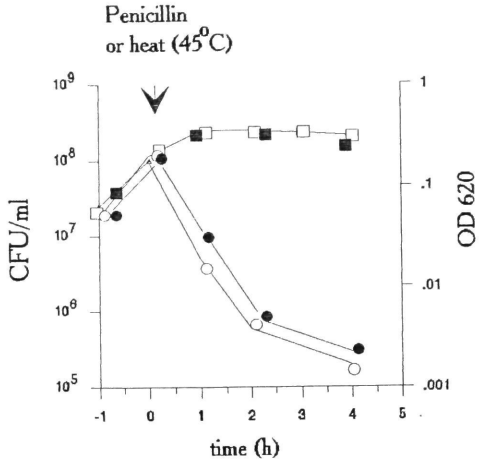
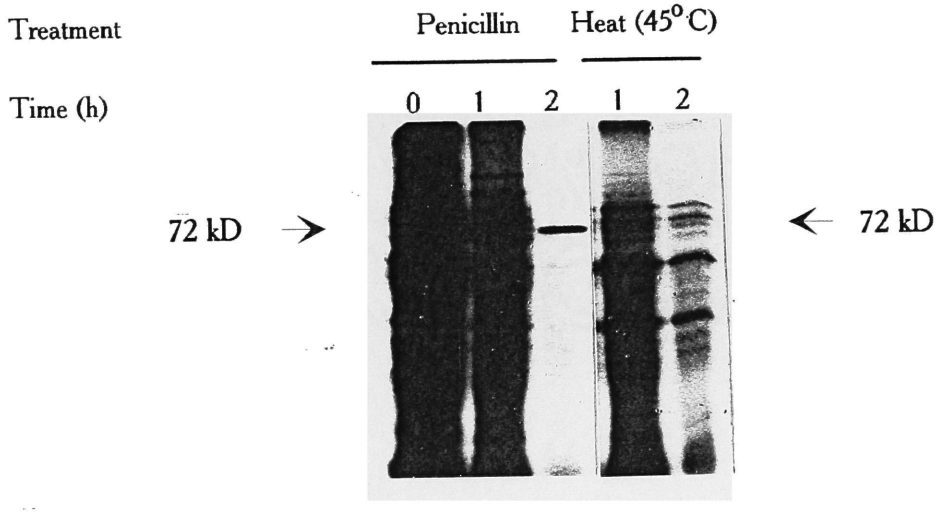

Antibiotic (20xMIC)
Penicillin
Chloramphenicol
Treatment duration (h)
0 2 3 4 1 2 3 4
72 kD
72 kD
Antibiotic (20xMIC)
Erythromycin
Rifampicin
Treatment duration (h)
0 1 2 3 4 1 2 3 4
72 kD
72 kD
Antibiotic (20xMIC)
Mitomycin
V Ctx Str
Treatment duration (h)
0 1 2 3 4 3 3 3
72 kD
72 kD

Figure 23: **The 72 kD protein was not produced during heat-shock conditions (45°C) which resulted in similar bacterial killing than treatment with 20x the MIC of penicillin. Cultures of Lyt 4-4 in the exponential phase of growth were treated either with 20x MIC of penicillin (open symbols) or switched to 45°C (closed symbols). OD_{620} (squares) and loss of viable counts (circles) in the cultures were followed (panel A). At several time-points during the treatment, samples were removed from the cultures, radioactively pulse-labeled and processed for separation by SDS-PAGE and autoradiography as described (panel B). Lanes were loaded with similar amounts of total proteins.**

A.


Penicillin
or heat (45°C)
CFU/ml
OD 620
time (h)

B.

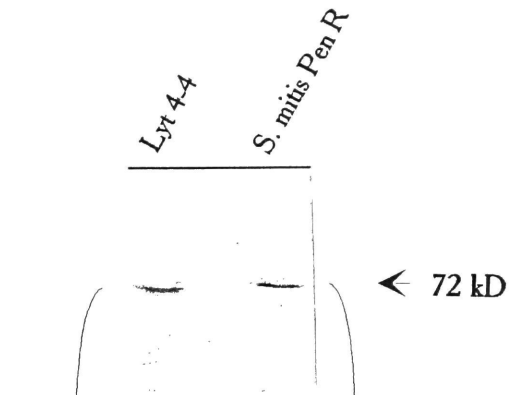

Treatment
Penicillin
Heat (45°C)
Time (h)
0 1 2 1 2
72 kD
72 kD

(v) Labeling of the 72 kD protein in other organisms. A protein similar to the 72 kD pneumococcal protein was also labeled during penicillin treatment of a strain of penicillin resistant *Streptococcus mitis*. Figure 24 (p. 129) shows that penicillin treatment of both Lyt 4-4 and *S. mitis* resulted in similar inhibition of global protein synthesis and production of a 72 kD radiolabeled protein, which produced identical peptide patterns after partial digestion with V8 protease.

igure 24: **Treatment of the penicillin resistant *S. mitis* PenR with 20x MIC of penicillin for 2-3 h also resulted in a global shutoff of protein synthesis and labeling of a unique band that migrated in parallel to the pneumococcal 72 kD on 10% SDS-PAGE (panel A). The similarity between the two bands was further assessed by identical peptide maps by partial proteolysis with V8-protease (panel B). The samples were handled and processed as described in figure 19.**

A.

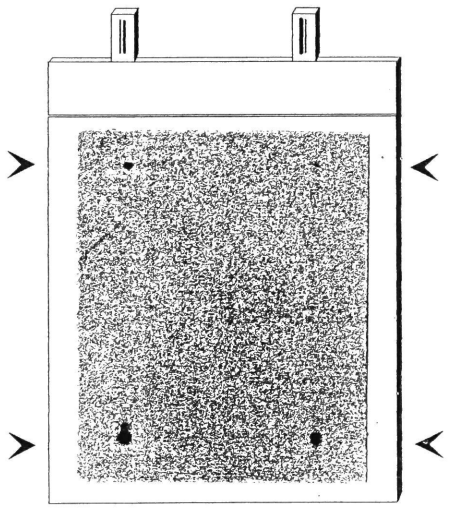
Cut slice containing the 72 Kd protein and load sideways on V8-protease containing gel.



B.

V8 protease containing stacking gel

15% SDS- PAGE



Discussion

Two striking observations emerged from the basic pulse-labeling experiment described in this chapter (see figure 15, p. 112). First, it appeared that both in Cid^+ and in Cid^- pneumococci the decrease in RNA and protein synthesis occurred with very similar initial rates during penicillin treatment, in spite of substantial differences (ca. 100x) in bacterial survival. Second, both types of bacteria induced and/or continued the specific radioactive labeling of a unique 72 kD protein with [^{35}S]-cysteine, even when incorporation of radioactive amino acids in of all other protein species had come to a virtual halt.

Inhibition of protein synthesis by penicillin treatment has first been suggested by Hotchkiss (36), in an elegant series of experiments where he biochemically determined the fate of the nitrogen atoms of amino acids (provided in the medium) in penicillin-treated or untreated cultures of *Staphylococcus aureus*. However, clear data on bacterial survival were not provided, and it was difficult to assess whether inhibition of protein synthesis was due to some sophisticated effect of the antibiotic or merely to bacterial death. Penicillin-induced inhibition of macromolecule synthesis was better demonstrated in later experiments (63), using radioactive precursors and a penicillin-tolerant strain of *Streptococcus mutans* (i.e., a bacterium which was inhibited but neither killed nor lysed by the drug). It was shown that penicillin treatment resulted in the

shutoff of both RNA and protein synthesis, and also, to a lesser extent, in diminished synthesis of DNA.

Since inhibition of protein synthesis renders bacteria resistant to killing and lysis by cell wall inhibitors, it was tempting to make some mechanistic association between (i) the ability of tolerant bacteria to shutoff protein synthesis in response to the antibiotic and (ii), their capacity to resist the bactericidal effect of the drug. It was suggested that tolerant bacteria might more readily shutoff macromolecule synthesis than non-tolerant cells during antibiotic treatment, in a manner reminiscent of the stringent response of RelA$^+$ *E. coli* to amino acid starvation (24 A).

In our experiments, we did not find striking differences between the initial rates of shutoff of macromolecule synthesis in penicillin-treated Cid$^+$ and Cid$^-$ cultures. Therefore, the increased survival of Cid$^+$ over Cid$^-$ pneumococci was not likely to be due to a more "stringent" response of the former than the latter organism. On the other hand, the almost identical decline of both RNA and protein synthesis during penicillin treatment of Cid$^-$ and Cid$^+$ cultures supported the proposition of Mychajlonka et al. (63), that some "talk-back" mechanism(s) might exist between the peripheral process of cell wall synthesis and the intracellular environment.

Details of this putative signal transduction mechanism are not known. However, some clue concerning the mediators of this signaling pathway might be drawn from previous experiments. For

instance, the fact that neither protoplasts nor stabilized L-form of bacteria (which may contain at least some penicillin binding proteins; (48)) are inhibited by penicillin suggest that the PBPs are not the primary mediators of the downregulation of macromolecule biosynthesis by the drug. The PBPs, however, are necessary, because they are responsible for the assembly of the cell wall, the presence of which is essential for the full inhibitory response to cell wall inhibitors. It is possible that some cell wall precursor or metabolite is the primary mediator of this so-called "talk-back" mechanism. The second element, is presumably a membrane bound sensor that might perceive the presence of the primary mediator and transmit a signal to a secondary intra-cellular effector. This hypothetical mechanism is analogous to the regulation ß-lactamase production in *B. licheniformis* and *E. coli* (41,87).

Beside the improbable assumption of a more stringent response in Cid^- than Cid^+ penicillin-treated pneumococci, another appealing hypothesis was that both types of cells might produce qualitatively or quantitatively different sets of "stress" proteins during penicillin treatment. It has been recently shown in *E. coli*, that co-expression of up to five heat shock proteins (68,95) interfered with lysis induced either by the bacteriophage ΦX174 E protein or by treatment with ß-lactam antibiotics. It is noteworthy, however, that heat shock proteins did not entirely protect the cells from killing by these agents. In other experiments, Jablonski et al. (39) showed that the

protein complement of a tolerant strain of *Staphylococcus aureus* was altered in the course of treatment with the ß-lactam antibiotic oxacillin. They suggested that the drug might induce the synthesis of alternative "stress" proteins. However, the observations were based on silver-stained gels of whole bacterial proteins (rather than on autoradiograms of pulse-labeled cells) and it was unclear whether the changes in protein profiles were due to *de novo* synthesis of some "stress" proteins or to processing and/or degradation of preexisting polypeptides.

Our experiments revealed an intriguing and totally unexpected phenomenon: the appearance of a cysteine-incorporating protein species, under experimental conditions, when synthesis of all other proteins had come to a virtual halt. The 72 kD protein could not be labeled by other amino acids and was not turned off by treatment of the cells with chloramphenicol. The 72 kD protein may be a polypeptide that undergoes post-translational modification through the addition of cysteine molecules that may occur through a non-ribosomal mechanism, like certain peptide antibiotics which are produced by multifunctional enzyme complexes (such as, for instance, in penicillin synthesis (71)). Enzyme-mediated synthesis of a short polypeptide has also been recently observed in *Streptomyces coelium* (92).

The function of the 72 kD labeled protein is unknown. Since it was labeled in response to several antibiotics resulting in halt of bacterial growth and polymer synthesis, it is possible that the 72 kD species is involved in the regulation of the

coordinate shutoff of cellular metabolism. One argument for such a possibility is that labeling of the protein was also regulated: it decreased after removal of penicillin from the medium, while bacterial growth and synthesis of other proteins resumed.

In conclusion, we used the isogeneic Cid^+ and Cid^- pneumococci that were both resistant to penicillin-induced lysis (Lyt^-) to look into early biochemical events that follow the covalent binding of the penicillin molecule to its cellular protein targets. Our observations clearly eliminate the often quoted hypothesis that bacterial death is related to a synthetic imbalance between the synthesis of cell wall (which is inhibited) and cytoplasmic mass (which was assumed to continue). In fact, the opposite was observed: penicillin treatment has lead to a rapid and complete shutoff in the synthesis of all - but one - protein species through a mechanism that appeared to be a reversible and global regulatory process. The unique 72 kD protein that incorporates [^{35}S]-cysteine may be part of this regulatory circuit

Appendix I

Additional characteristics of the Cid$^-$ mutant T6

During the characterization of the Cid$^-$ mutant T6, it was found that growth of the organism in the chemically defined medium Cden (either in liquid cultures or on agar plates) required the addition of at least 0.02% (w/v) of yeast extract (the supplementation used in most experiments was 0.1%). Since slow bacterial growth can affect susceptibility to penicillin-induced killing, it was necessary to test whether yeast extract was a growth limiting factor which might be responsible for the Cid$^-$ phenotype. This was not the case, because supplementation of growing cultures of Cid$^-$ cells with 10x (1%) the standard amounts of yeast extract did not influence the Cid$^-$ phenotype.

The requirement for yeast extract (Y$^-$) in Cden appeared to be genetically linked to the Cid$^-$ phenotype. Competent Cid$^-$ (Y$^-$) pneumococci (strain T6) were transformed with DNA of the Cid$^+$ (Y$^+$) donor strain Lyt 4-4 and selected for the ability to grow on Cden agar plates lacking yeast extract (Y$^+$ phenotype). The frequency of transformation to the Y$^+$ phenotype was 0.1 to 1%, which was identical to that of a control point mutation (Nov) marker. When tested for penicillin-induced killing, all 50 independent Y$^+$ transformants had regained the Cid$^+$ phenotype along with the Y$^+$ character. In contrast, none of 15 Y$^-$ control colonies were Cid$^+$. Conversely, when Cid$^+$ cultures were

transformed with Cid$^-$ DNA and submitted to enrichment cycles with penicillin, the appearance of Cid$^-$ cells was paralleled by that of Y$^-$ bacteria.

The linkage between Cid$^-$ and Y$^-$ allowed us to examine the rate of reversion of Cid$^-$ (Y$^-$) to Cid$^+$ (Y$^+$). "Young" (18-20 h old) colonies of Y$^-$ cells were suspended in tubes containing 1 ml of rich medium (Cden + Y), incubated at 37°C and culture growth was followed by plating samples in duplicate on Cden agar plates supplemented or not supplemented with 0.1% of yeast extract. Figure 25 (p. 138) shows that Y$^-$ cells displayed a 3 h lag phase upon transfer into liquid medium. The Y$^+$ revertant were detected at a frequency of approximately 10^{-4}-10^{-5} during growth in liquid cultures. However, while both Y$^-$ cells and Y$^+$ revertants grew at virtually identical rates, neither the revertants nor wild-type pneumococci displayed a 3 h lag phase of growth when grown in liquid cultures. Complementary experiments showed that this difference in lag phase was responsible for the progressive loss of the Cid$^-$ (Y$^-$) phenotype after multiple freezing and regrowth of an original Cid$^-$ (Y$^-$) culture.

We attempted to determine the nature of the factor required for growth of Y$^-$ pneumococci from the composition of the yeast extract (Difco) provided by the factory. When supplemented in purified form (either individually or in different combinations), none of the known components of the extract supported growth of Y$^-$ cells. Therefore, the essential substituent probably belonged to the unidentified portion of yeast extract. Preliminary ultra-

filtration experiments show that the molecular weight of this unidentified factor is between 500 and 1000 Daltons.

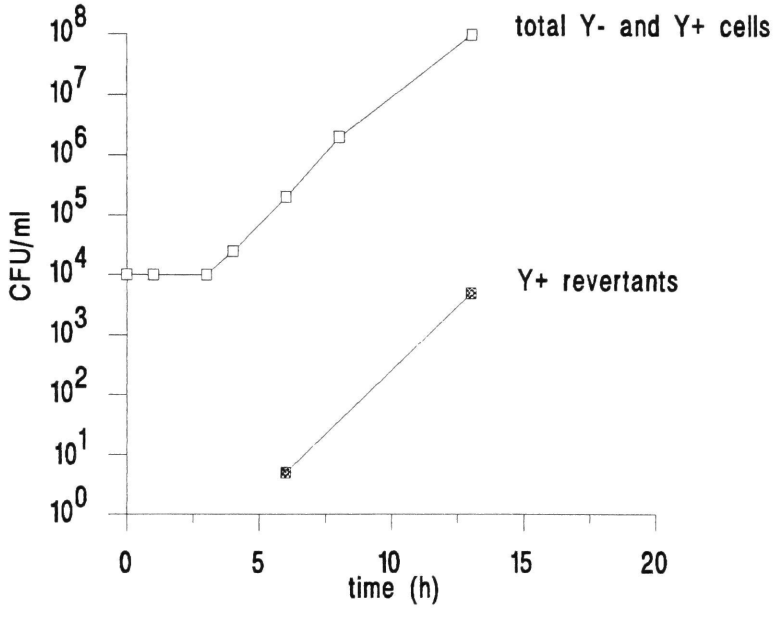


Figure 25: **Reversion of Y^- (Cid^-) cultures to the Y^+ (Cid^+) phenotype. Young colonies (18-20 h) of the Y^- (Cid^-) strain T6 were suspended in 1 ml of rich medium (Cden + Y) and incubated at 37°C. At several time-points during the incubation, dilutions of the cultures were plated in duplicate on Cden agar supplemented with 0.1% of yeast extract (allowing growth of both Y^- and Y^+ cells; open symbols) or not supplemented with yeast extract (only allowing growth of Y^+ revertants; closed symbols). Upon regrowth in new cultures, the Y^+ revertants did not show the 3 h lag phase in bacterial growth observed in Y^- cells. Y^+ revertants also had regained the Cid^+ phenotype.**

Appendix II

Control of pneumococcal *lytA* gene conversion by an heterologous plasmid delivery system.

The experiment is summarized in figure 26 (p. 140). The *E. coli* plasmid pGL80, containing the cloned pneumococcal *lytA* gene (14), was expanded in *E. coli* DH5α and used to transform (convert) the Lyt⁻ Erm pneumococcal mutant RUP-24 (*lytA::em*; see table 1, p. 25) to the Lyt⁺ phenotype. Transformants were first screened for DOC lysis, and then tested for erythromycin resistance.

Figure 26 shows that the results can be of two types. First, if single cross-over (Campbell-like) recombination occurs, the Lyt⁺ transformants should contain both the *lytA* and the *lytA::em* alleles (as a result of insertion-duplication) and their phenotype should be Lyt⁺ Erm. Second, if double cross-over (gene conversion) takes place, the Lyt⁺ transformants are expected to have lost the Erm marker, due to the conversion of their *lytA::em* allele by the cloned *lytA* gene. The results showed that the recombination event was essentially of the second type: all of nine Lyt⁺ independent transformants had lost their Erm marker while acquiring the functional *lytA* gene. This confirmed that gene conversion (double cross-over) was more frequent than insertion-duplication (single cross-over) in this system, and that pneumococci could process DNA expanded in *E. coli* DH5α.

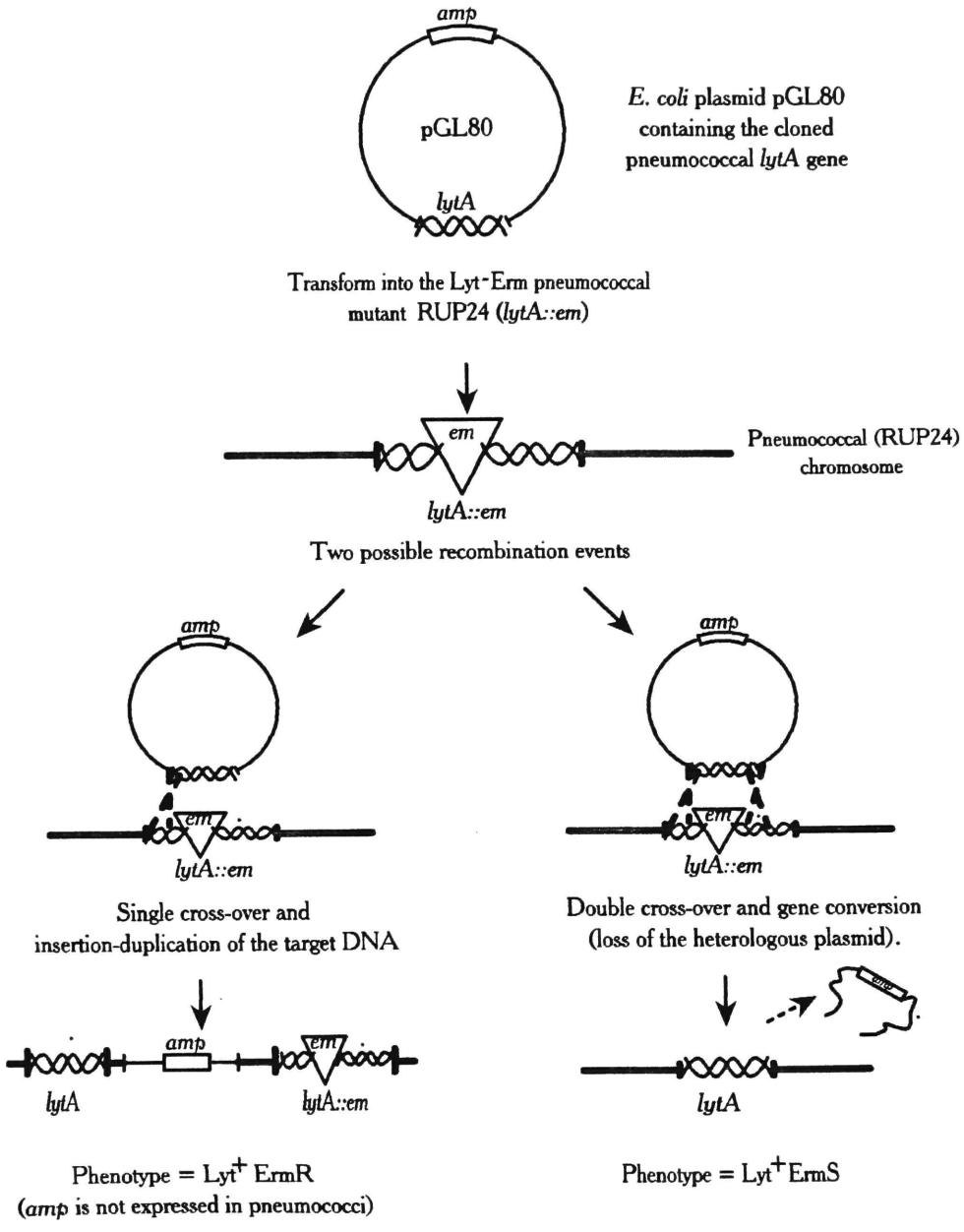


Figure 26: Control of insertion-duplication versus gene conversion using the heterologous plasmid pGL80, containing the cloned pneumococcal *lytA* gene (see text for details)

Appendix III

Analysis of cell wall stem peptides by high performance liquid chromatography (HPLC).

Compared to susceptible strains, penicillin-resistant pneumococci (with altered PBPs) often contain major alterations in the stem peptides network (cross-link) of their peptidoglycan (19,21). We have tested whether the Cid^- phenotype might also be associated with such type of alterations. Cell walls from both Cid^+ Lyt^- (Lyt 4-4) and Cid^- Lyt^- (T6) pneumococci were purified as described, digested with amidase and submitted to HPLC analysis using published methods (18,20).

In brief, 5-10 mg of purified cell walls/ml (in 50 mM of HEPES pH 7 containing 0.1% of Brij and 0.05% of NaN_3) were digested overnight at 37°C with approximately 20 units of amidase/mg. The products of the digestion were dried, and the residual buffer was extracted with acetone. The precipitate was dried and the peptides were extracted with acetone-isopropanol-water (25:25:50) containing 0.1% of trifluoroacetic acid (TFA). The solvent were removed by evaporation and the peptides were redissolved in 0.1% of TFA for analysis.

The chromatographic system was made up of a 721 system controller, two 510 pumps, a U6K injector and a 730 data module from Waters Associates, Inc. (Milford, MA). The detector was a variable-wavelength ISCO V4 detector with a 6-mm path length and

a 3.5 μl illuminated volume flow cell (ISCO, Lincoln, NE). The column used was a Vydac 218TP54 (The Separations Group, Hesperia, CA), and the eluting solvent was a 100 min linear gradient from 0.1% TFA (Pierce Chemicals Co., Rockford IL) to 15% acetonitrile (Burdick & Jackson, Muskegon, MI) in 0.1% of TFA pumped at a flow rate of 0.5 ml/min. The eluted fractions were detected by their A_{210}, at which wavelength the amide bonds are the main chromophores.

The results (figure 27, p. 143) show the absence of significant difference in the major peaks of the chromatograms.

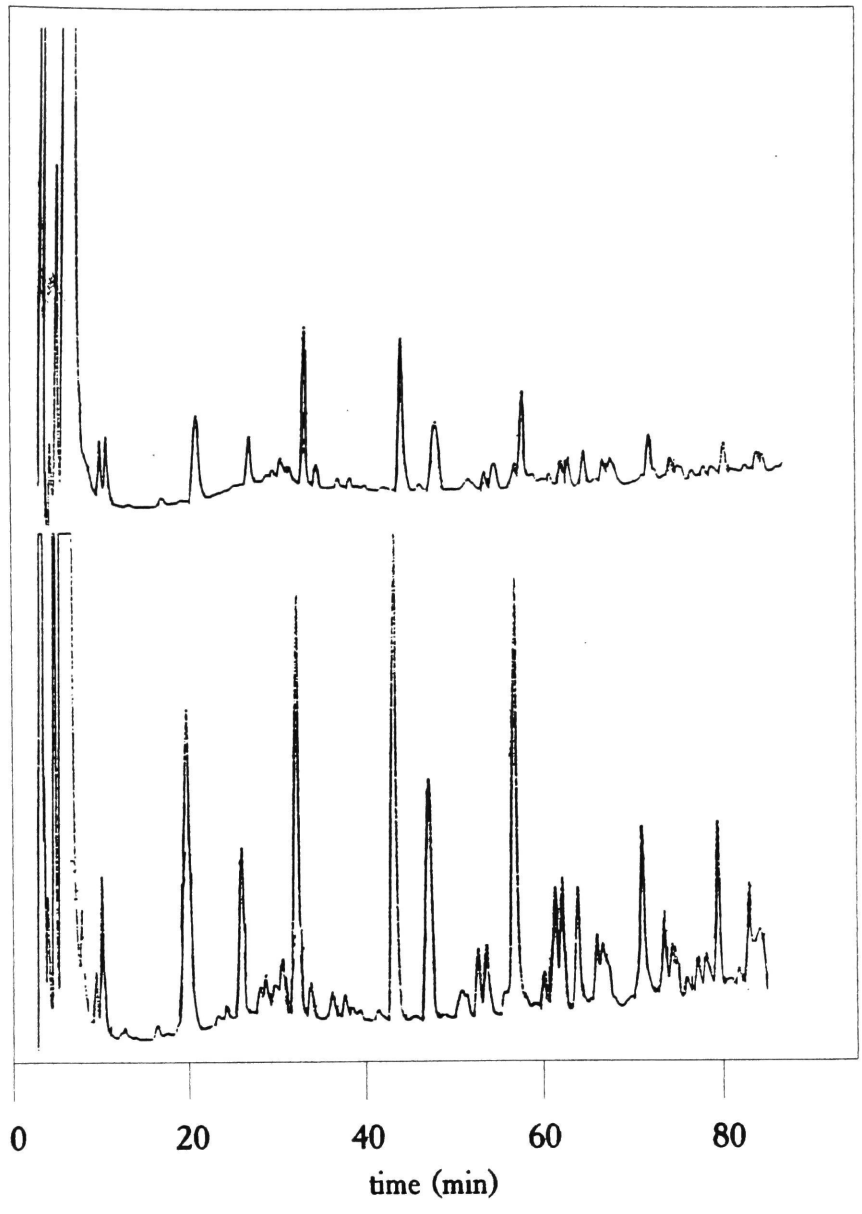


Figure 27: Purified cell walls of the Cid^+ Lyt^- strains Lyt 4-4 (upper chromatogram) and the Cid^- Lyt^- strain T6 (lower chromatogram) were digested with amidase and the stem peptides were extracted and separated by reverse-phase HPLC. The eluted fractions were detected by their A_{210}. Approximately 3x less material was loaded on the column for Lyt 4-4 (upper chromatogram) than for T6 (lower chromatogram).

Appendix IV

Construction of pneumococcal mutants producing increased amounts of a "nicking" enzyme activity.

In these experiments, we used the method of gene duplication mutagenesis described in chapter II (see figure 7, p. 58). A complete *EcoR*1 digest of the wild-type strain R6x was ligated at the homologous site of the "suicide" vector pVA891 and transformed back in competent cells of R6x. Transformants were selected for Erm (the marker of the "suicide" vector) and screened for increased rates of penicillin-induced lysis.

Two clones (Lys-2 and Lys-30) out of 240 Erm transformants tested, were lysed faster than the parent R6x when challenged with 20x the MIC of penicillin. The "superlyser" phenotype (called Lys-2) cotransformed with the Erm marker in backcross experiments, and was not due to alterations of either the cell wall or the production of amidase, as assessed by the techniques described in chapter III. However, when transformed in either of the Lyt$^-$ mutants Lyt 4-4 or Rup-27 (*lytA::em::cm*) the Lys-2 mutation resulted in a new phenotype: the cultures lost optical density at an increased rate when challenged with 20x the MIC of penicillin (figure 11, p. 89 in chapter III). This increased lysis rate was due to a greater number of breaks (or nicks) in the cell walls, as compared to control Lyt$^-$ cultures. The breaks could be visualized by electron microscopy (see insets in figure

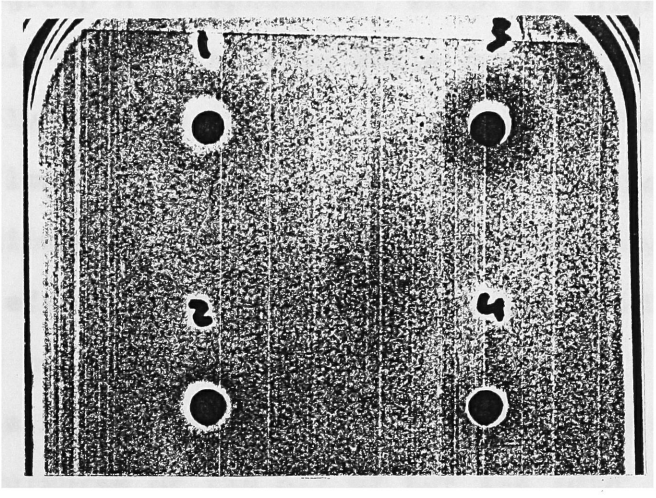
11, p. 89) and did not result from cell wall solubilization, because Lys-2 mutants radiolabeled in their cell walls did not release the radioactive tracer during lysis (as opposed to amidase-induced lysis).

Cell extracts of the Lys-2 mutants contained an increased enzymatic activity that could lyse both (i) *Micrococcus lysodeikticus* cell walls (a substrate which is resistant to digestion by the pneumococcal major autolysin amidase; see figure 28, p. 146) and (ii) Lyt$^-$ pneumococci, when added to the cultures along with penicillin (data not presented).

The "nicking" enzyme, overproduced by the mutants, might be the analog of the "second" murein hydrolyse (a glycosidase) recently described by Lopez et al. (17,74). However, in Lys-2 mutants, overproduction of the enzyme did not increase the rate of penicillin-induced killing (see figure 11). This observation casts new doubts about the importance of "nicking" enzymes in the cidal effect of penicillin on pneumococci.

Figure 28: **Lysis of *Micrococcus lysodeikticus* cells by pneumococcal extracts. Proteins extracted by LiCl treatment of partially purified pneumococcal cell walls were tested for their ability to lyse *M. lysodeikticus* cells embeded in agarose (Kallestad Quantiplate kit, Chaska, Mn). Equal amounts of proteins of the extracts were loaded in the wells. The strains were R6x (Lyt^+), Lys-2 (Lyt^-; fast-lyser), RUP-24 (Lyt^-) and T6 (Lyt^-). The plates were incubated for 48-72 h at 37^oC.**

R6x Lys-2 mutant



Rup-24 T6

Appendix V

Degradation of RNA during penicillin treatment of Cid$^+$ and Cid$^-$ pneumococci.

In group A streptococci, which are highly susceptible to penicillin-induced killing while notably resistant to antibiotic-induced lysis (see figure 2, p. 16 in introduction), penicillin-induced loss of viability has been associated with increased degradation of cellular RNA (56). It was suggested that the killing effect of penicillin might be mediated by some enhanced nuclease activity in the bacteria.

This hypothesis was tested in Cid$^+$ and Cid$^-$ pneumococci. In brief, cultures of (10 ml) of Lyt 4-4 (Cid$^+$ Lyt$^-$) and T6 (Cid$^-$ Lyt$^-$) grown in Cden minus uridine (but supplemented with 0.1% of yeast extract) were labeled for about 6 generation-times with [^{3}H]-uridine (specific activity and concentration as described in chapter IV). At an OD_{620} of 0.15, the cultures were thoroughly washed 2x (by centrifugation) resuspended in nonradioactive (prewarmed) medium and further incubated (chased) at 37°C. At an OD_{620} of 0.25, 20x the MIC of penicillin was added and optical density and viable counts were followed. At several time-points before and after the addition of penicillin, 500 μl samples of the cultures were removed and either (i) TCA-precipitated (for 30 min on ice) by the addition of 4.5 ml of 10% ice-cold TCA, or (ii) diluted with 4.5 ml of water and boiled for 10 min (Mc

Dowel, personal suggestion). The samples were then filtered through glass microfiber filters (Whatman) saturated with a solution of cold uridine (1 mg/ml). The filters were thoroughly rinsed with cold uridine-containing water, dried and the radioactivity was counted in a scintillation counter as described.

The results are depicted in figure 29 (p. 149). No significant difference in loss of high molecular weight radiolabeled RNA was observed between Cid^+ and Cid^- cells, either in TCA precipitates or in boiled samples (not shown of the graph).

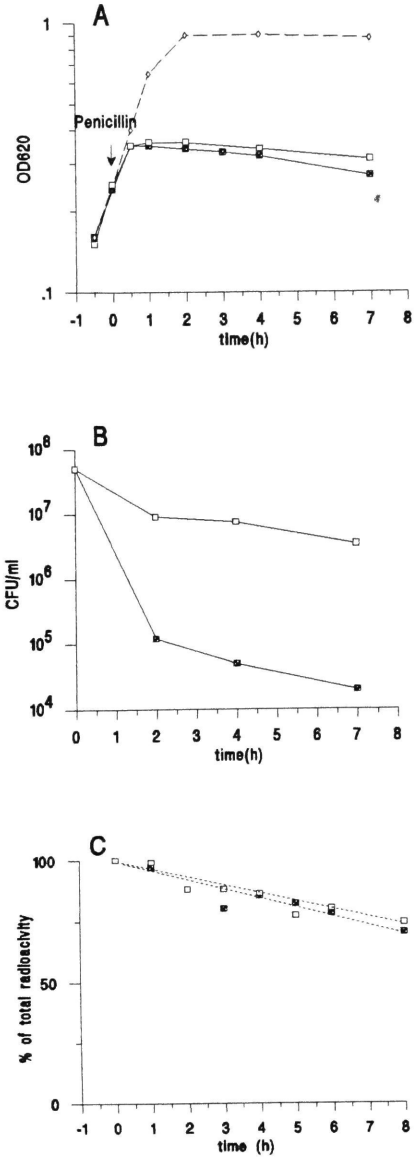


Figure 29: **Degradation of RNA during penicillin treatment of Cid^+ and Cid^- pneumococci. Cultures of Lyt 4-4 (Cid^+ Lyt^-; closed symbols) and T6 (Cid^- Lyt^-; open symbols) were pulse-labeled with [^{3}H]-uridine, chased with cold precursors and treated with 20x the MIC of penicillin. Optical density (panel A), viable counts (panel B) and loss of TCA precipitable radioactivity RNA (panel C) were followed. The dashed line in panel A indicate the growth curve of control, untreated cultures.**

Appendix VI

Survival of penicillin-induced pneumococcal protoplasts.

The most compelling evidence for the primary role of penicillin-induced lysis in the bactericidal effect of penicillin relies on the classic *E. coli* K-12 spheroplast experiments by Lederberg (47). When bacteria were suspended in an osmotically protective medium (containing sucrose and magnesium) and treated with penicillin, the cells rapidly converted to wall-less spheroplasts, that could still perform some vital functions (such as protein synthesis), and were able to resume growth after reversal to rod-shaped cells in drug-free medium. In contrast, penicillin-treated cultures in hypo-osmolar medium lysed and died. Hence, it was important to test whether osmotic protection might also prevent penicillin-killing in pneumococci.

Among several different conditions that were tested, we found that pneumococci could be grown (although growth rate was slow) and converted to stable protoplasts in C+Y medium supplemented with 30% of raffinose. Protoplast formation and stability were tested both by phase-contrast microscopy and OD_{620}, and the ability of penicillin-induced protoplasts to resume growth was evaluated by subculturing dilutions of the master culture (from 10^{-2} to 10^{-8}) into tubes containing the same protective medium supplemented with 1,000 U of penicillinase.

R6x was grown in C+Y supplemented or not supplemented with

raffinose and treated with 20x the MIC of penicillin at an OD_{620} of 0.25. In the protective medium, sixty percent of the cells had converted to protoplasts 30 min after addition of the drug and >95% after 1 h. The protoplasts were stable for up to 20 h and even resulted in some increase in optical density (but not viability). In contrast, R6x grown in non-protective C+Y was rapidly lysed within 2 h of penicillin treatment (see figure 30, p. 152). However, in spite of osmotic protection, pneumococcal protoplasts rapidly lost viability (figure 30 C).

It was possible that the inability of protoplasts to resume growth was not due to alterations of the protoplasts themselves, but rather to the absence of some cell wall "primer" necessary to resume wall synthesis. Therefore, the experiments mentioned above were repeated with the Lyt^- strain Lyt 4-4. The results showed that while Lyt 4-4 did not lose their cell walls and did not convert to protoplasts, the cultures lost viability at almost the same rates as R6x. Therefore, the maintained presence of the cell wall did not provide any more protection against penicillin-induced killing in these conditions (figure 30 D).

Thus, in contrast to experiments in *E. coli* K-12, osmotic protection could not prevent penicillin-induced killing in pneumococci, suggesting that differences exist between the two systems.

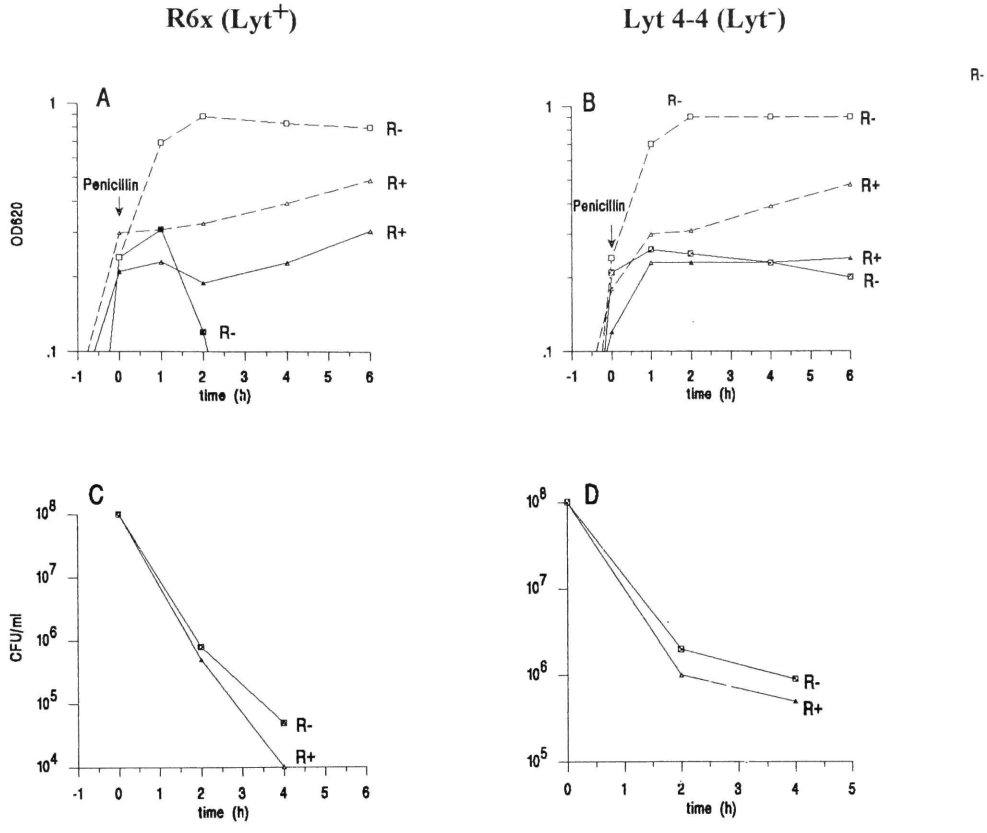
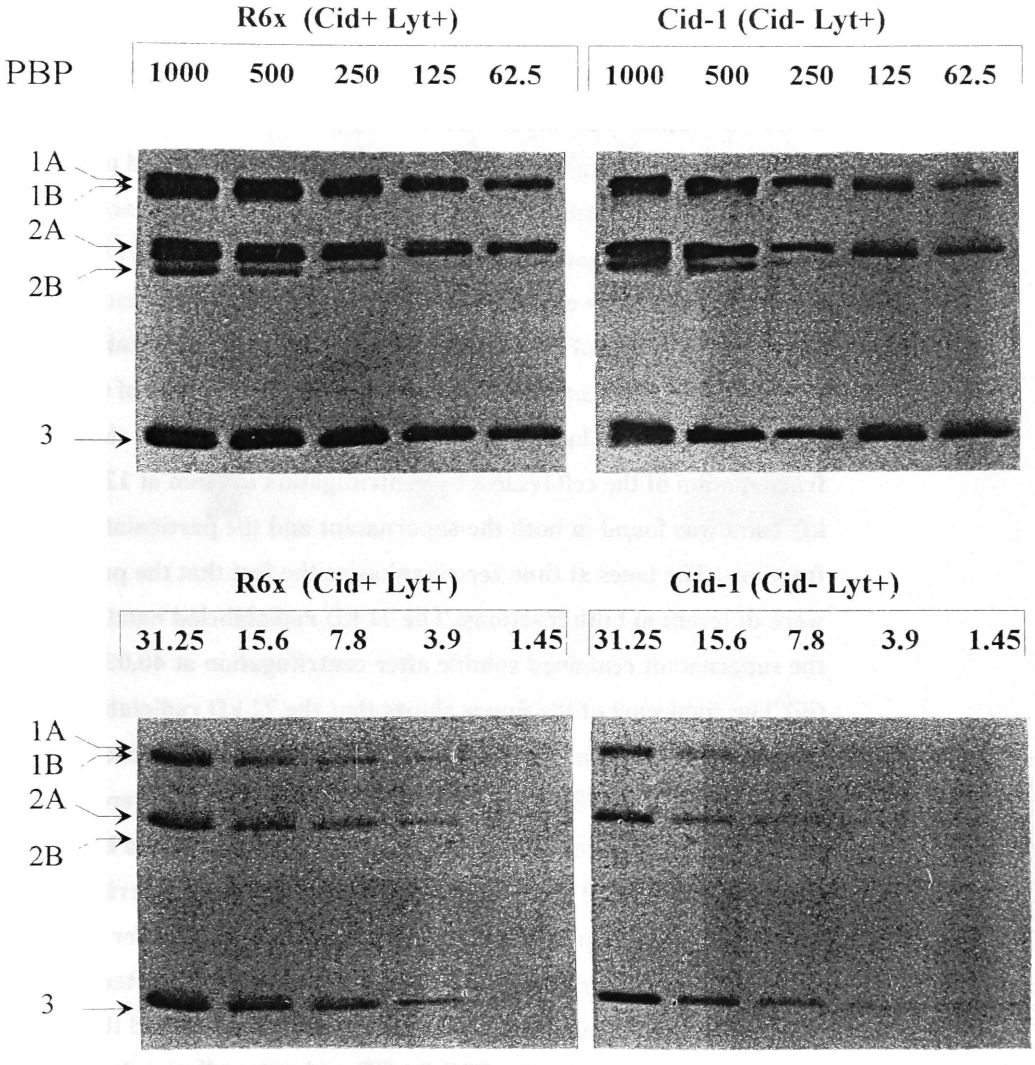


Figure 30: **Survival of penicillin-induced stable protoplasts of pneumococci. Cultures of either R6x (Lyt$^+$) or Lyt 4-4 (Lyt$^-$) were grown in plain C+Y (R-) or in C+Y supplemented with 30% of raffinose (R+). One portion of the cultures received 20x the MIC of penicillin (continuous lines) whereas the other portion was used as a control (dashed lines). Optical density (panels A and B) and viable counts (panels C and D) were followed. For determination of viable counts, dilutions of the cultures were inoculated in tubes containing raffinose-supplemented C+Y and penicillinase. Protoplast formation was assessed by phase contrast microscopy. In cultures of R6x, > 95% of the bacteria had converted to protoplasts within the first hour of penicillin treatment. In contrast, Lyt 4-4 cultures did not convert to protoplasts during penicillin treatment, whether or not they had been grown in high raffinose C+Y.**

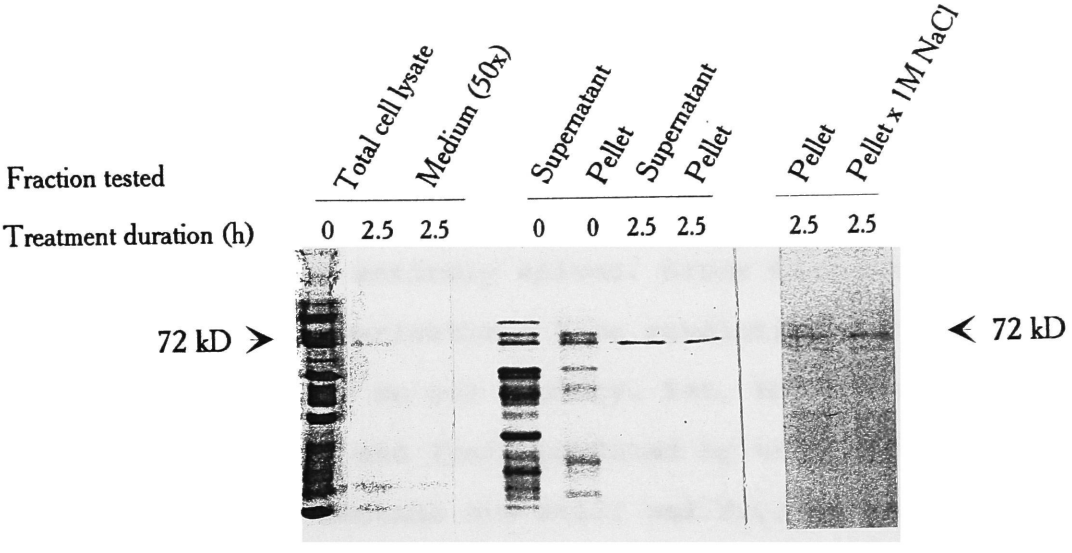
Appendix VII

Figure 31: **Determination of the molecular sizes and the penicillin-binding affinities of the PBPs of strain R6x (Cid^+ Lyt^+) and of the mutant Cid-1 (Cid^- Lyt^+). Cultures in the exponential phase (1 ml) were incubated with [^{3}H]-penicillin at concentrations ranging from 1-1000 ng of antibiotic/ml for 10 min at 37°C. An excess of unlabeled penicillin was then added and the samples were immediately chilled on ice. The cells were recovered by centrifugation at 12,000 g for 5 min at 4°C, suspended in 50 ul of 50 mM sodium phosphate buffer (pH 7.0) with 1% of the detergent Sarkosyl and incubated for 15 min at 37°C. This treatment resulted in the dissolution of the microorganisms. Techniques used for discontinuous gel electrophoresis and fluorography were as described (93). Equal amounts of total proteins were on the gel. The figure shows that PBPs of both the Cid^+ strain R6x and the Cid^- mutant Cid-1 migrated identically on SDS-PAGE and had comparable affinities for radioactive penicillin as shown by the intensities of the radioactively labeled band on the autoradiograms. Note that there was an overlap between PBP 1A and 1B on the autoradiogram at high concentrations of radioactive penicillin (250-1000 ng/ml). At lower concentrations, PBP 1B is not seen because it has a lower affinity for penicillin than PBP 1A.**


R6x (Cid+ Lyt+)
Cid-1 (Cid- Lyt+)
PBP
1000 500 250 125 62.5
1000 500 250 125 62.5
1A
1B
2A
2B
3
R6x (Cid+ Lyt+)
Cid-1 (Cid- Lyt+)
31.25 15.6 7.8 3.9 1.45
31.25 15.6 7.8 3.9 1.45
1A
1B
2A
2B
3

Appendix VIII

Figure 32 : **Detection of the 72 kD radiolabeled protein in different fractions of the cultures or the cell lysates by autoradiography on the PhosphorImager (left part of the figure) or on X-OMAT films (center and right part of the figure). Cultures of the Cid^+ Lyt^- strains Lyt 4-4 in the mid-logarithmic phase of growth were treated with 20x the MIC of penicillin and pulse-labeled with Tran^{35}Slabel, as described, before being processed for autoradiography. (i) The left part of the figure shows that the 72 kD radiolabeled protein was essentially absent from the growth medium. Radiolabeled bacteria were pelleted by centrifugation and the proteins present in the cell-free supernatant were precipitated (overnight with 10% TCA at 4°C) and resuspended in a 50x smaller volume of 50 mM KPO_4 buffer at pH 7.5. Bacteria were lysed enzymatically (with amidase) as described. Equal amounts of total protein were loaded in each lanes. (ii) The central part of the figure shows that after fractionation of the cell lysates by centrifugation (30 min at 12,000 g), the 72 kD band was found in both the supernatant and the particulate (pellet) fractions. The lanes at time zero emphasize the fact that the protein profiles were different in both fractions. The 72 kD radiolabeled band recovered from the supernatant remained soluble after centrifugation at 40,000 g for 30 min. (iii) The right part of the figure shows that the 72 kD radiolabeled band was not released from the particulate (membrane-enriched) fraction by treatment with 1 M NaCl at 37°C for 30 min. Membranes were recovered by serial centrifugation as follows: bacterial lysates were first cleared by low-speed centrifugation (4,000 g x 5 min) followed by high speed centrifugation (40,000 g x 30 min) and the pellets were resuspended in KPO_4 buffer (50 mM, pH 7.5) supplemented or non supplemented with 1 M NaCl. After 30 min of incubation at 37°C, the samples were centrifuged again and the pellets were processed for separation by SDS-PAGE and autoradiography. Equal amounts of proteins were loaded in each lanes. Note that experiments in which NaCl was replaced by LiCl gave similar results (not shown on the figure).**


Fraction tested
Total cell lysate
Medium (50x)
Supernatant
Pellet
Supernatant
Pellet
Pellet
Pellet x 1M NaCl
Treatment duration (h)
0 2.5 2.5 0 0 2.5 2.5 2.5 2.5
72 kD
72 kD

Summary and conclusion

The first report by Fleming on a mold-produced factor (i.e., penicillin) that was capable of lysing and killing *Staphylococcus aureus* appeared in the British Medical Journal in 1929. The first clinical report on the usefulness of penicillin in severe bacterial infections (in war wounds) appeared 14 years later in the Lancet, a time at which the molecular structure of the new drug had not been entirely solved. Since that time, penicillin and its ß-lactam derivatives have revolutionized the impact of bacterial diseases on our society. Yet, the phenomena of bacterial killing and lysis produced by treatment of susceptible bacteria with ß-lactams are still not fully understood. The subject of the present thesis was to reexamine these phenomena and design new experimental approaches to better understand the mechanism(s) of penicillin-induced killing and lysis in pneumococci.

In the early fifties, the nature of the bacterial cell wall as an anatomically and molecularly distinct part of the bacterium was demonstrated. At about the same time, the diphospho-uridine-linked muropeptides were discovered in penicillin-treated cells (the Park peptides). In the mid-fifties, Lederberg observed the change of penicillin-treated cells of *E. coli* from rod-shaped bacilli to osmotically fragile spheroplasts (47). He proposed that the target of penicillin was the so-called cell wall (just discovered). In the sixties, first Martin (52A) and then Tipper

and Strominger (77) proposed that the mechanism of action of penicillin was based on its structural similarity to the ubiquitous D-alanyl-D-alanine termini of the precursors of the bacterial cell wall peptidoglycan. From the mid-sixties to the mid-seventies, the importance of transpeptidases and carboxypeptidases (located in the plasma membrane) for the synthesis of peptidoglycan was recognized. In the following decade, the family of penicillin-binding proteins (or so-called penicillin-interactive proteins) was discovered. This led to the characterization of the molecular targets of the ß-lactam molecules, eventually leading to the cloning, classification, and solving of the molecular structure of the proteins by X-ray crystallography as well as to the identification of the active site residues interacting with the ß-lactam molecule (43,67).

However, as to how treatment of bacteria with penicillin leads to its most impressive effect (i.e., killing and lysis) remained unclear. An early hypothesis was based on the interpretation of an aspect of selectivity of the drug, namely inhibition of cell wall synthesis. The abrupt disintegration of the bacteria after the addition of penicillin apparently without interference with the mass increase of the culture has led to the intuitive notion that killing and lysis were due to the disruption of a weakened cell wall (inhibited in its synthesis) by the combined mechanical and osmotic pressure of an expanding (non-inhibited) cytoplasmic mass. In this model, killing was equated to lysis (84).

With the discovery of murein hydrolases (autolysins) in the second half of the sixties, the proposal was expanded by Weidel et al. (91 A) as an "imbalanced growth", i.e., an imbalance between lytic and synthetic activities of cell wall related enzymes. More specifically, it was proposed that introduction of "nicks" (by autolysins) in the preexisting cell wall was an essential part of the cell wall synthetic mechanism, which allowed new wall building blocks to be incorporated. The hypothesis was that inhibition of cell wall synthesis (without inhibition of autolysis) would automatically lead to the well known phenomena of lysis and killing.

The discovery of antibiotic tolerance in autolysin-defective pneumococci (80) provided experimental proof for part of this hypothesis, i.e., autolysin-defective cells indeed did not lyse with penicillin, but retained a normal MIC value. However, subsequent studies with the same system led to the recognition that the model proposed by Weidel was not be fully correct. It appeared that the autolysin involved in penicillin-induced lysis of pneumococci (i.e., amidase) was <u>not</u> essential for cell wall synthesis and was dispensable for bacterial growth as shown in bacteria with a number of genetic alterations in the amidase determinant (*lytA*), including deletion of the gene (6,81). Thus, in the case of pneumococci, penicillin-induced lysis was not mediated by an imbalance between the activities of synthetic and lytic enzymes, but rather by the deregulated activity of an autolysin (amidase) that was not required for synthesis of a

structurally "normal" cell wall peptidoglycan.

For these reasons, it was not possible any longer to assume that penicillin treatment would automatically lead to enzymatic cell wall damages. It was proposed that penicillin treatment "triggered" an abnormal cell destructive activity of these enzymes, through some regulatory defect(s) introduced by penicillin into the bacteria. A specific version of this model proposed that the key event in the model may be the release of autolysin inhibitors (lipoteichoic acids or LTA) into the medium or the dissociation of an intracellular autolysin-inhibitor complex in the penicillin-treated cell (82,84).

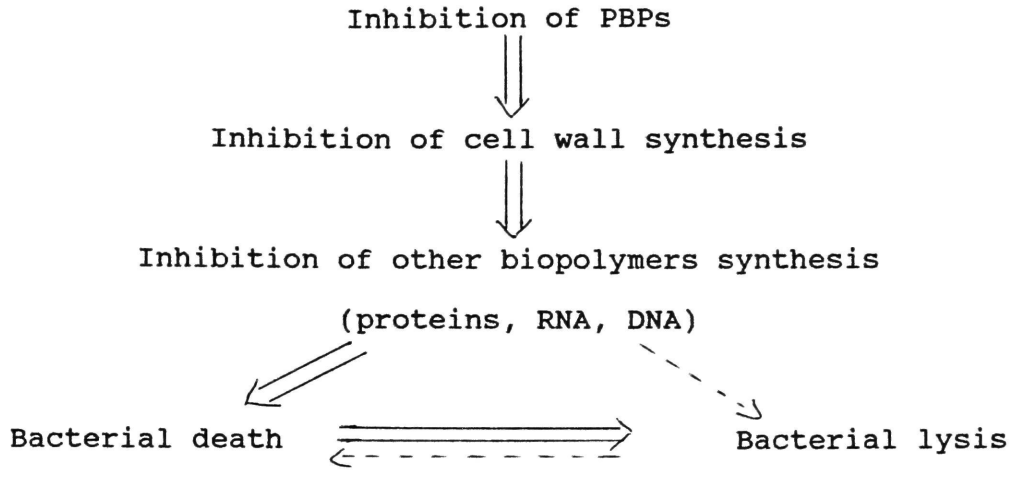
Although the continued cidal activity of penicillin in autolysin mutants had been noted (80,84), the slowdown in the killing effect of penicillin in lytic mutants led to the widely held belief that the mechanism of killing was the autolytic disintegration of the bacteria. The starting point of this thesis was the reexamination of this question. Summaries and interpretations of the experiments were given at the end of each Chapter of the thesis. Here, I shall briefly recapitulate the main findings and conclusions of this work.

The experiments described in this thesis have clearly shown that in pneumococci (and likely also in other bacteria) penicillin-induced killing occurs by at least two distinct mechanisms, (i) one which is autolysis-dependent and accounted for the loss of about 1 Log unit CFU/ml of culture and (ii) a second mechanism, which is independent of autolysis by the major

autolysin amidase and was responsible for the loss of 3-4 Log units CFU/ml of culture.

The central element of this second mechanism was a genetic locus (provisionally called the *cid* locus), which was not linked to the amidase gene (*lytA*) that is responsible for the classic phenomenon of autolysis. Genetic experiments in which the *cid*$^-$ mutation and the *lytA* gene were brought together in a single cell also revealed that the *cid*$^-$ mutation had at least a partial controlling effect on the triggering of autolysis by penicillin.

The examination of the cidal and lytic activities in this context also suggested that the activity of the *cid* determinant (i.e., killing) precedes activation of autolysis. These experiments allow one to suggest a sequence of events, which begin with the specific binding and inhibition of the membrane-bound PBPs by penicillin, most likely followed in a rapid succession by an abnormal course of wall synthesis (94), production of a regulatory signal(s) which shuts down biopolymer synthesis; a "killing event" (resulting in the loss of viability) and - finally - triggering of autolysin activity. A simplified scheme of such a sequence of events is given on the next page.



These experiments are consistent with the model proposed earlier that the primary effect of penicillin is growth inhibition (84).

The construction of Cid$^-$ Lyt$^-$ double mutants has allowed for the first time to examine early events in the sequence of responses following the inhibition of penicillin-binding proteins. Addition of penicillin to these bacteria, which were not lysed and suffered only a minor decline in viable titers, resulted in a rapid and massive shut down in the synthesis of polypeptides. At the same time, a single and unique 72 kD polypeptide species began and/or continued to incorporate radioactive [^{35}S]-cysteine. These surprising and novel observations may be the first evidence for the existence of a regulatory circuit between the biosynthesis of cell wall and the rest of biopolymer synthesis. As discussed in Chapter IV (p. 131-2), such a system would involve a primary mediator (made of some cell wall precursor or metabolite) and a presumably membrane-bound sensor which might perceive the presence of the primary

mediator and transmit a signal to an intracellular effector. The 72 kD protein may be part of this circuit and may represent a new antibiotic-induced stress factor. It is of significance that both the global shutoff and the labeling of the 72 kD protein were also observed in other non-lysing streptococcal species.

Extensive comparative experiments were performed using isogeneic pairs of Cid^+ Lyt^- and Cid^- Lyt^- pneumococci, in an attempt to define the nature of the killing event(s). No differences were found between these strains in terms of the complement of their penicillin-binding proteins, muropeptide composition of their cell walls, sensitivity of their cell walls to the autolysin amidase, the rates with which cellular proteins and RNA synthesis decreased after addition of penicillin and the number and nature of protein components resolvable by 2-D gel electrophoresis.

Extensive genetic approaches to clone and sequence the *cid*$^-$ gene have proved so far unsuccessful. Neither shotgun mutagenesis based on gene insertion-inactivation nor cloning in *E. coli* produced the expected gene, in spite of the fact that appropriate control experiments proved the adequacy of the experimental systems. It is probably significant, in this respect, that attempts to clone genetic elements responsible for similar phenotypes have encountered the same difficulties in *Staphylococcus aureus* and in *E. coli*. The appearance of a new nutritional requirement in the Cid^- mutant used in our experiments and the close genetic linkage between these two

markers suggest that null-mutants in the *cid* determinant may be lethal because of a normal physiological function of this gene. Thus, the biochemical nature of the penicillin-induced killing event in bacteria remains a mystery.

On the basis of more complex experiments we propose that the *cid* locus product(s) may operate at the level of the plasma membrane and might have some analogy with the bacteriophage lytic systems (96). Specifically, the Cid protein(s) may have a similar function to the S-protein of lambda phage. It is proposed that the penicillin-induced overproduction (or deregulation) of the Cid protein(s) would lead to membrane damage in a manner similar to the channel- (or holin) forming bacteriophage encoded S-protein aggregates. In Lyt^- cells, the membrane damage *per se* would be responsible for most of the killing. In the presence of autolytic enzymes (i.e., in Lyt^+ bacteria), the Cid protein(s) would control (directly or indirectly) the access of these enzymes to the bacterial cell wall, thus advancing the phenomenon of viability loss to autolysis.

In order to explain the unique ability of cell wall inhibitors to trigger this sequence of phenomena, we propose that the expression of the Cid protein(s) is under negative control by a repressor, which may be composed of some cell wall material, cell wall precursors, or some other metabolite (LTA; soluble, uncrosslinked peptidoglycan oligomers), the synthesis of which is perturbed in the penicillin-treated cell. In this model, the well known suppression of the cidal and lytic effects of penicillin by

inhibition of protein synthesis also requires a new interpretation: the mechanism is not the prevention of the increase in bulk cytoplasmic mass, but rather the prevention of overexpression of a specific (hypothetical) toxic protein(s) (see figure 13, p. 98).

The kind of cyclic antibiotic exposure used to produce Cid$^-$ mutants in the laboratory was very similar to the kind of antibiotic pressure operative in the clinical environment, at least with the classical ß-lactams which have relatively short half-lives (58). Interestingly, a very large proportion of clinical isolates of penicillin-resistant pneumococci have been shown to carry traits (resistance to penicillin-induced lysis) similar to traits in mutants constructed in the laboratory (49,58). In the case of *Enterococcus faecalis*, cyclic exposure to penicillin has led to lysis and kill resistant bacteria. While *Enterococcus faecalis* from the pre-penicillin era would be lysed and killed during penicillin treatment, the kill and lysis resistance to penicillin is a universal property of most contemporary isolates of *Enterococcus faecalis*, suggesting that in this particular species, penicillin treatment in the clinical environment may have introduced the penicillin-tolerance trait in these bacteria (32A).

The acquisition of a phenotype enabling bacteria to survive antibiotic treatment has an obvious selective advantage. In addition, carrying the antibiotic-tolerance trait may make tolerant cells preferred recipients in genetic exchanges

involving antibiotic resistance genes. The frequent association of decreased penicillin-induced lysis (tolerance) and penicillin resistance (i.e., the ability of bacteria to grow in spite of the presence antibiotic in the medium) in clinical isolates of pneumococci may be the result of such a sequence of events (i.e., tolerance followed by resistance) in the bacteria (49,58).

The observations and conclusions of this thesis set the stage for future experimentations in this field and it may be worthwhile to briefly enumerate key areas that need further exploration.

As stated above, the nature of the penicillin-induced killing event(s) is not known. The experiments with the osmotically stabilized protoplasts suggested that an irreversible membrane damage might cause loss of viability. It is relevant, in this regard, that penicillin treatment of bacteria may lead to the secretion (or shedding) of a number of membrane-associated molecules, including LTA, phospholipids and proteins (see 3,34,67A,82). It is also possible, however, that the second pneumococcal autolysin, capable of "nicking" the glycan chain acts in tandem with the activity of the Cid protein(s) at the membrane. The experiments with the insertion-duplication mutant (Lys-2) overproducing this second enzyme showed that it is the rate of autolysis, and not the bactericidal susceptibility that is primarily affected in these mutants. This finding is consistent with the model in which the event determining the rate of killing is the number of wall "nicks" per cell rather then

their size.

Further characterization of the Cid$^-$ mutants will require approaches that are complementary to those presented in this thesis. First, on the biochemical and physiological levels, it will be important to test whether Cid$^+$ and Cid$^-$ cells differ in their membrane and/or wall content in LTA as well as in the kinetics of secretion of LTA (and other membrane-related molecules) during penicillin treatment. LTA is a membrane-bound molecule which (among other functions) has the capability to strongly inhibit autolysin-induced cell wall degradation (3,35,67A,82). Therefore, it is possible that LTA might be implicated in the inhibition of cell wall degradation by amidase in Cid$^-$ cells.

Second, the molecular characterization of the *cid* gene (or locus) is critical because it might give us the opportunity to investigate both the *cid* gene product(s) itself as well as the organization and the control of the *cid* element(s) in the bacterial genome. From the negative results of the experimental systems presented in this thesis, it is clear that the cloning strategies will have to be different in order to avoid inactivation and overexpression of the gene. Because of the potential importance of this molecular genetic approach, it may be worthwhile to spell out available alternative cloning strategies in some detail.

Cloning in *S. pneumoniae* could be attempted by a "gene rescue" system using the technique of insertion-gene duplication

mutagenesis with an heterologous plasmid carrying an Erm marker as described in Chapter II (see figure 7, p. 58). First, libraries (in the heterologous vector) containing either the *cid* or the *cid*$^-$ allele should be created. Second, such libraries would be used to transform recipient cells containing the other allele of the gene (e.g., bacteria containing the *cid*$^-$ mutation - which also confers the Y$^-$ phenotype - will be transformed with libraries containing the *cid* allele - conferring the Y$^+$ phenotype). Third the transformants selected for insertion of the heterologous plasmid (by the acquisition of the plasmid's Erm marker) could be screened for the parallel reversion of the Y$^+$ or Y$^-$ phenotype (e.g., Y$^-$ to Y$^+$ in the example mentioned above). Reversion of the phenotype in this situation would suggest dominance of the wild-type gene (conferring Y$^+$) over the mutated allele (conferring Y$^-$). The gene may then be cloned by analyzing the DNA adjacent to the heterologous vector using standard molecular genetic techniques.

Alternatively, the *cid* gene might be cloned by generating libraries of pneumococcal DNA in an *E. coli* bacteriophage. This system would avoid overexpression of the target DNA in a multicopy plasmid. Several techniques (not described here) are available to recover DNA directly from phage plaques in order to test its ability to transform either the Cid$^+$/$^-$ or the Y$^+$/$^-$ phenotypes into adequate pneumococcal recipient cells.

It would also be important to create additional Cid$^-$ mutants: either conditional mutants or mutants altered in other

putative genes affecting penicillin-induced killing, which might be less difficult to clone.

Finally, as to the characterization of the 72 kD protein labeled by radioactive-cysteine, a first attempt to sequence a purified peptide fragment resulting from a partial digestion with V8 protease has failed for technical reasons (the N-terminal of the peptide was blocked). Purification of the whole protein (as well as of other peptide fragments) is currently underway. Determination of a partial sequence of the protein will allow further investigations at the levels of both the biochemistry and the molecular biology of this intriguing polypeptide.

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