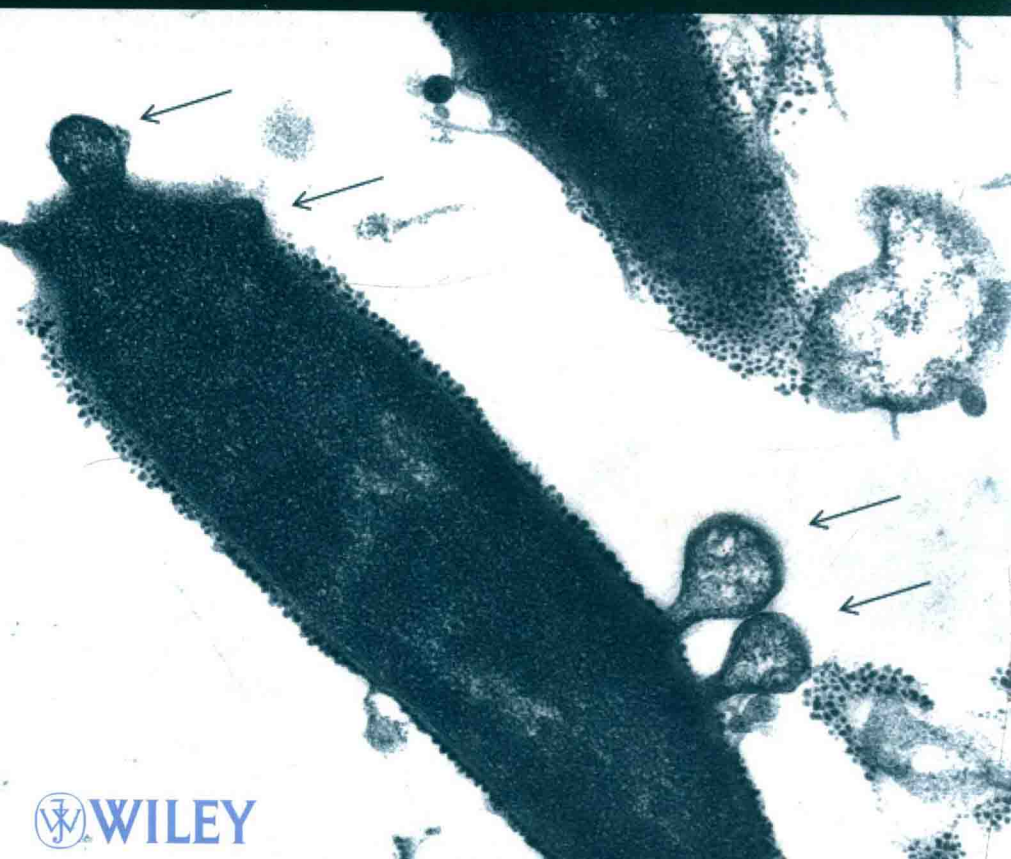
Enzybiotics

Antibiotic Enzymes as Drugs and Therapeutics

Edited by
Tomas G. Villa
Patricia Veiga-Crespo



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Antibiotic Enzymes as Drugs and Therapeutics

Edited by

TOMAS G. VILLA
School of Biotechnology
University of Santiago de Compostela

and

PATRICIA VEIGA-CRESPO
Department of Microbiology
Faculty of Pharmacy
University of Santiago de Compostela

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PREFACE

The writing of a preface on an old-yet-new subject, such as the one in this book, is always a difficult task. It is common knowledge that the discovery of penicillin, and subsequently the rest of the antibiotics, has probably been one of the most important scientific contributions to civilization. By saving millions of lives, antibiotics automatically increased the half-life of mankind, thus allowing scientists to give their best to society for 20 to 30 additional years.

It is also common knowledge that resistance to antibiotics is a constant possibility and unfortunately something to be considered every time a new antibiotic goes on the market. Because of this, and because the discovery and design of new antibiotics becomes more and more difficult every year, society, through the work of several worldwide research groups, is looking into the use of what one of us (Dr. Vincent Fischetti) has termed "enzybiotics" (the result of blending the words "enzymes" and "antibiotics"), for treating bacterial and fungal diseases, either alone or in combination with antibiotics.

The book starts with four chapters in which the potential, advantages, and phylogeny of enzybiotics are reviewed. Then, the new ways of controlling infections by Gram-negative bacteria and an updated view of bacteriophage holins are presented. After a review of anti-staphylococcal lytic enzymes, the book goes on to discuss membrane-targeted enzybiotics, as well as the design of phage cocktails for current therapy. Finally, the last two chapters deal respectively with the novel methods to identify new enzybiotics and the use of modified phages to induce suicide in bacteria.

All in all, the contributors are all active researchers, involved in the topic of enzybiotics. It is hoped that the joining of different points of view, such as those reflected in this book, will help to clarify the

emerging field of enzybiotics and to consolidate the idea that the therapies mediated by these compounds may contribute to the relief of pain and to the control of contagious diseases.

Santiago de Compostela, Spain
December 24, 2008

TOMAS G. VILLA
PATRICIA VEIGA-CRESPO

CONTRIBUTORS

Juan C. Alonso Department of Microbial Biotechnology, Centro Nacional de Biotecnología, CSIC, 28049 Madrid, Spain; jcalonso@cnb.csic.es or jcalonso@cnb.uam.es

Jan Borysowski Department of Clinical Immunology, Transplantation Institute, Warsaw Medical University, Poland; jborysowski@interia.pl

Vincent A. Fischetti Professor and Head Laboratory of Bacterial Pathogenesis, Rockefeller University, 1230 York Avenue, New York, NY 10021, USA; vaf@rockefeller.edu OR vaf@mail.rockefeller.edu

María Gasset Instituto de Química-Física "Rocasolano," Consejo Superior de Investigaciones Científicas, Serrano 119, 28006, Madrid, Spain; mgasset@iqfr.csic.es

Lawrence Goodridge Department of Animal Sciences, Colorado State University, Fort Collins, CO 80523, USA; Lawrence.Goodridge@ColoState.edu

Andrzej Górski Department of Clinical Immunology, Transplantation Institute, Warsaw Medical University, and Laboratory of Bacteriophages, L. Hirszfeld Institute of Immunology and Experimental Therapy, Wrocław, Poland; agorski@ikp.pl

Jonathan E. Schmitz Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, 1230 York Avenue, Box 172, New York, NY 10065, USA; jschmitz@rockefeller.edu

Raymond Schuch Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, 1230 York Avenue, Box 172, New York, NY 10065, USA

Marcelo E. Tolmasky Center for Applied Biotechnology Studies, Department of Biological Science, College of Natural Science and Mathematics, California State University Fullerton, Fullerton, CA 92834-6850, USA; mtolmasky@Exchange.fullerton.edu

Patricia Veiga-Crespo Department of Microbiology, Faculty of Pharmacy, University of Santiago de Compostela, Spain; patricia.veiga@usc.es

Tomas G. Villa Department of Microbiology, Faculty of Pharmacy, University of Santiago de Compostela, and School of Biotechnology, University of Santiago de Compostela, Spain; tomas.gonzalez@usc.es

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CHAPTER 1

ENZYBIOTICS AND THEIR POTENTIAL APPLICATIONS IN MEDICINE

JAN BORYSOWSKI[1] and ANDRZEJ GÓRSKI[1,2]
[1]Department of Clinical Immunology, Transplantation Institute, Warsaw Medical University, Poland
[2]Laboratory of Bacteriophages, L. Hirszfeld Institute of Immunology and Experimental Therapy, Wrocław, Poland

1. INTRODUCTION

Over the last decade, a dramatic increase in the prevalence of antibiotic resistance has been noted in several medically significant bacterial species, especially *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Klebsiella pneumoniae*, as well as *Staphylococcus aureus*, coagulase-negative staphylococci, enterococci, and *Streptococcus pneumoniae* (Hawkey 2008). This unfavorable situation is further aggravated by a shortage of new classes of antibiotics with novel modes of action that are essential to contain the spread of antibiotic-resistant pathogens (Livermore 2004). In fact, some infectious disease experts have expressed concerns that we are returning to the pre-antibiotic era (Larson 2007). Therefore, there is an urgent need to develop novel antibacterial agents to eliminate multidrug-resistant bacteria (Breithaupt 1999). A very interesting class of novel (at least in terms of their formal clinical use) antibacterials are enzybiotics.

The term "enzybiotic" was used for the first time in a paper by Nelson et al. (2001) to designate bacteriophage enzymes endowed with bacterial cell wall-degrading capacity that could be used as antibacterial agents. While some authors suggest that this name should refer to all enzymes exhibiting antibacterial and even antifungal activity (Veiga-Crespo et al. 2007), in this chapter we will discuss only bacterial cell wall-degrading

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enzymes (regardless of their source). Other names that are used with respect to enzybiotics are lytic enzymes and peptidoglycan hydrolases. The latter refers to the major mode of action of enzybiotics, that is, the enzymatic cleavage of peptidoglycan covalent bonds, which results in the hypotonic lysis of a bacterial cell. Peptidoglycan hydrolases constitute an abundant class of enzymes and may be obtained from different sources, for instance, bacteriophages (lysins) and bacteria themselves (bacteriocins and autolysins). Yet another example of well-known enzybiotics are lysozymes, including hen egg white lysozyme and human lysozyme (a list of representative enzybiotics is shown in Table 1.1).

In view of the ever-increasing antibiotic resistance of bacteria, the most important characteristics of enzybiotics are a novel mode of antibacterial action, different from those typical of antibiotics, and the capacity to kill antibiotic-resistant bacteria (Borysowski et al. 2006). Another significant feature of some lytic enzymes is the low probability of developing bacterial resistance (in some cases, the development of enzybiotic resistance results in a reduction in bacterial fitness and virulence; Kusuma et al. 2007).

The goal of this chapter is to discuss the major groups of enzybiotics, including lysins, bacteriocins, autolysins, and lysozymes, in the context of their potential medical applications.

2. LYSINS

2.1. General Features

Lysins or endolysins are double-stranded DNA bacteriophage-encoded enzymes that cleave covalent bonds in peptidoglycan (Borysowski et al. 2006; Fischetti 2008). They are naturally produced in phage-infected bacterial cells during the course of lytic cycle. At the last stage of the cycle, endolysin molecules degrade peptidoglycan, thereby causing lysis of the bacterial cell and ensuring the release of progeny virions (Young et al. 2000). The term "endolysin" was introduced to the scientific literature by F. Jacob and C. R. Fuerst to stress that enzyme molecules act on peptidoglycan from within the bacterial cell in which they are synthesized (Jacob and Fuerst 1958). In view of this, it appears that recombinant enzymes acting on the cell wall from outside the cell (e.g., those used for therapeutic purposes) should be referred to as lysins rather than endolysins. Still another name proposed to designate a lysin is "virolysin," which is intended to point out the viral origin of these enzymes (Parisien et al. 2008). However, this name has not gained popularity and is used very rarely.

TABLE 1.1. A List of Representative Enzybiotics

Enzybiotic Name	Enzybiotic Class	Source	Enzymatic Specificity	Antibacterial Range	Reference
PlyC	Lysin	Phage C1	Amidase	*S. pyogenes*, groups C and E streptococci	Nelson et al. 2006
Pal	Lysin	Phage Dp-1	Amidase	*S. pneumoniae*	Loeffler et al. 2001
Cpl-1	Lysin	Phage Cp-1	Muramidase	*S. pneumoniae*	Loeffler et al. 2003
PlyGBS	Lysin	Phage NCTC 11261	Endopeptidase muramidase	*S. agalactiae*, groups A, C, G, L streptococci	Cheng et al. 2005
Phage B30 lysin	Lysin	Phage B30	Endopeptidase muramidase	*S. agalactiae*, groups A, B, C, E, G streptococci, *E. faecalis*	Baker et al. 2006
LambdaSa1 prophage lysine	Lysin	Prophage LambdaSa1	Endopeptidase	?	Pritchard et al. 2007
LambdaSa2 prophage lysine	Lysin	Prophage LambdaSa2	Endopeptidase glucosaminidase	*S. pyogenes, S. dysgalactiae*, group E streptococci, *S. equi*, group G streptococci, *S. agalactiae*	Pritchard et al. 2007
PlyG	Lysin	Phage γ	Amidase	*B. anthracis*	Schuch et al. 2002
PlyL	Lysin	Prophage λBa02	Amidase	*B. cereus* *B. anthracis*	Low et al. 2005
PlyPH	?	?	?	*B. anthracis*	Yoong et al. 2006
PlyB	Lysin	Phage BcpI	Muramidase	*B. anthracis*	Porter et al. 2007
Ply118	Lysin	Phage A118	Peptidase	*Listeria*	Loessner et al. 2002
Ply500	Lysin	Phage A500	Peptidase	*Listeria*	Loessner et al. 2002

TABLE 1.1. ***Continued***

Enzybiotic Name	Enzybiotic Class	Source	Enzymatic Specificity	Antibacterial Range	Reference
Ply3626	Lysin	Phage Ø3626	Amidase	*C. perfringens*	Zimmer et al. 2002
PlyV12	Lysin	Phage Φ1	Amidase	*E. faecalis, E. faecium, S. pyogenes*, group B, C, E, G streptococci	Yoong et al. 2004
Lyt A	Autolysin	*S. pneumoniae*	Amidase	*S. pneumoniae*	Rodriguez-Cerrato et al. 2007
lysostaphin	Bacteriocin	*S. simulans*	Endopeptidase	*S. aureus*, coagulase-negative staphylococci	Patron et al. 1999
zoocin A	Bacteriocin	*S. equi*	Endopeptidase	*S. equi, S. pyogenes, S. mutans, S. gordonii*	Simmonds et al. 1995
hen egg white lysozyme	Lysozyme	Hen's egg white	Muramidase	Gram-positive bacteria	Sava 1996
		Ø3626			et al. 2002

The table does not include staphylococcal phage lysins that are discussed in Chapter 7.

The main mode of antibacterial action of lysins is the enzymatic cleavage of the covalent bonds in peptidoglycan. Depending on their enzymatic specificities, lysins fall into five major classes: *N*-acetylmuramoyl-L-alanine amidases, endopeptidases, *N*-acetylmuramidases (lysozymes), endo-β-*N*-acetylglucosaminidases, and lytic transglycosylases (Fig. 1.1).

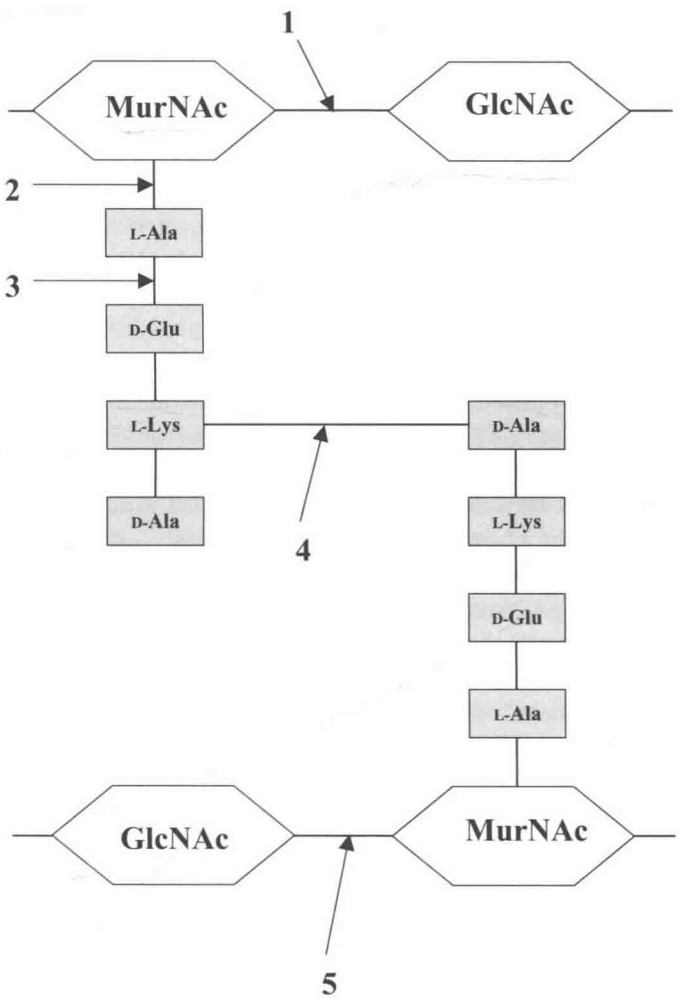

Figure 1.1. Sites of peptidoglycan cleavage by main classes of enzybiotics. This variant of peptidoglycan is typical of *S. aureus*. The backbone of peptidoglycan consists of alternating residues of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). The tetrapeptide side chains branching off from *N*-acetylmuramic acid are cross-linked by the pentaglycine bridges. The sites of cleavage by enzybiotics with different enzymatic specificities are indicated by the numbered arrows: (1) muramidases and transglycosylases; (2) amidases; (3 and 4) endopeptidases; (5) glucosaminidases.

The majority of lysins described to date exhibit only one kind of muralytic activity, whereas relatively few possess two separate enzymatic domains (Borysowski et al. 2006).

While the main mode of antibacterial activity of lysins is based on enzymatic cleavage of peptidoglycan, it is noteworthy that some of them can also affect bacterial cells by a nonenzymatic mechanism. This mechanism relies on destabilization of the bacterial cytoplasmic membrane by amino acid sequences whose properties, especially their amphipathic secondary structure, positive charge, and hydrophobicity, are similar to those found in cationic antimicrobial peptides (CAPs). Such sequences were identified in T4 phage lysozyme and lysins encoded by *Pseudomonas aeruginosa* phages D3 and ΦKZ (Düring et al. 1999; Rotem et al. 2006). In a series of elegant experiments, these sequences were shown to be more important for T4's antibacterial activity than the enzymatic cleavage of peptidoglycan (Düring et al. 1999). As mentioned above, sequences having physicochemical characteristics typical of CAPs (X1 and Z1) are also contained within lysins encoded by two *P. aeruginosa* phages (Rotem et al. 2006). It was shown that synthetic peptides with amino acid sequences corresponding to X1 and Z1, as well as their shorter analogs, inhibited the growth of several Gram-positive bacterial species in a mechanism analogous to that of CAPs. The authors of the study suggested that endolysins of other phages could also be a source of novel antimicrobial peptides. Another unusual enzyme is the *Bacillus amyloliquefaciens* bacteriophage auxiliary lysin lys1521, whose positively charged C-terminal sequences were shown to increase the permeability of the *P. aeruginosa* outer membrane, thereby facilitating the access of the N-terminal enzymatic domain to peptidoglycan (Muyombwe et al. 1999; Orito et al. 2004).

A typical feature of lysins is their modular structure, which means that they are composed of at least two distinct domains: an N-terminal catalytic domain and a C-terminal bacterial cell wall-binding domain (Loessner et al. 2002; Loessner 2005). As mentioned above, some lysins possess two different catalytic domains. In some lysins both the catalytic and the cell wall-binding domain are indispensable for their lytic activity, while others can lyse bacteria also in their C-truncated forms, although it is the C-terminal domain that is responsible for binding to the bacterial cell wall. Interestingly, lysins were also reported to exhibit higher antibacterial activity after removing their C-terminal domains. These findings are very important because they indicate that the antibacterial activity of some lysins could be increased by simply removing their cell wall-binding domains (Borysowski et al. 2006).

Another typical feature of the vast majority of lysins described to date is a narrow antibacterial range when acting on the bacterial cell from outside. This range is usually limited to the host bacterial species of the bacteriophage encoding the given enzyme. However, it needs to be stressed that lysins are most often capable of killing the majority of strains within a given bacterial species (Fischetti 2008). For instance, Pal, an amidase encoded by *S. pneumoniae* phage Dp-1, was shown to lyse 15 out of 15 pneumococcal strains tested (Loeffler et al. 2001). Another lysin, Ply3626 of *Clostridium perfringens* bacteriophage Ø3626, could also kill all of the 48 *C. perfringens* strains tested (Zimmer et al. 2002). This feature clearly sets lysins apart from lytic phages, which are usually capable of infecting and killing only a small subset of strains within a given bacterial species. Very few lysins were reported to possess a broader antibacterial spectrum (Yoong et al. 2004).

A unique medical application of lysins may be the specific elimination of pathogenic bacterial species (e.g., *S. aureus*) colonizing mucous membranes without adversely affecting normal microflora. Such bacteria can, in some clinical settings, be a starting point for infections (Bogaert et al. 2004; Wertheim et al. 2005). Lysins could thus provide a basis for a novel strategy for preventing some bacterial infections. Furthermore, elimination of the mucosal reservoir of bacteria could contribute to containing the horizontal spread of bacterial pathogens in some communities (Fischetti 2003). Lysins appear to be better decolonizing agents than antibiotics owing to their species-specific and rapid antibacterial activity, capacity for killing antibiotic-resistant bacteria, and lower risk of developing resistance (Fischetti 2003; Cheng et al. 2005). Moreover, a considerable body of experimental data shows that lysins, in spite of their apparent immunogenicity, may also be successfully used in the treatment of systemic bacterial infections and are in this regard effective even after repeated administration (Loeffler et al. 2003; Borysowski et al. 2006).

Discussed below are lysins specific to medically significant bacterial species, including *Streptococcus pyogenes* (group A streptococci), *S. pneumoniae*, *Streptococcus agalactiae* (group B streptococci), *S. aureus*, and *Bacillus anthracis*. However, it needs to be stressed that specific lysins can be most likely obtained for any Gram-positive bacterial pathogen from dsDNA bacteriophage (Schuch et al. 2002). Gram-negative bacteria are essentially resistant to recombinant lytic enzymes due to the presence of the outer membrane (see subsection 6.2). Of particular importance is that lysins are also capable of killing antibiotic-resistant bacteria, as shown for penicillin-resistant *S. pneumoniae*

(Loeffler et al. 2001), vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* (Yoong et al. 2004), as well as methicillin-resistant *S. aureus* (MRSA; O'Flaherty et al. 2005) and *S. aureus* strains with reduced susceptibility to vancomycin (Rashel et al. 2007). It was also shown that lysins can act synergistically with other lytic enzymes and antibiotics (Loeffler and Fischetti 2003; Djurkovic et al. 2005; Becker et al. 2008).

***2.1.1. Lysins Specific to* S. pyogenes** The first and hitherto only lysin specific to *S. pyogenes* that was evaluated as a potential antibacterial agent was PlyC amidase derived from group C streptococci C1 phage (Nelson et al. 2001; Nelson et al. 2006). This enzyme is very interesting in at least two respects. First, it is the most potent lysin reported so far, its activity being over two orders of magnitude higher than those of other bacteriophage lytic enzymes. Second, PlyC is the only known multimeric lysin, while all the others are synthesized as single polypeptides. Although PlyC was first reported in 1957, it was not until 2001 that its antibacterial activity was studied in more detail both *in vitro* and *in vivo*. In fact, it is the first lysin whose activity was studied with a view to potential prophylactic or therapeutic use. *In vitro* experiments revealed that, unlike C1 phage, the enzyme lyses *S. pyogenes* most efficiently, while its activity against groups C and E is substantially lower. All 10 *S. pyogenes* strains tested were efficiently lysed by PlyC. On the other hand, the lysin practically did not act on streptococci groups B, D, F, G, L, and N or other bacterial species with the exception of *Streptococcus gordonii*, which was lysed very slowly. Such an antibacterial range appears to be very advantageous because it is essentially limited to pathogenic streptococci (groups A and C). In a murine model of oral colonization, a single dose of the lysin administered to the oral cavity of mice prior to 10^7 colony forming units (cfu) of group A streptococci resulted in significant protection from the mucosal colonization (only 28.5% of the mice that received PlyC were colonized compared with 70.5% of the animals in the control group). Importantly, in most mice that were colonized despite administration of enzyme, cfu counts remained low throughout the experiment or the bacteria were completely eliminated within 48 h, whereas those in the control group increased during the same period of time. In another experiment, no streptococci were detected in oral swabs of nine heavily colonized mice 2 h after administration of one lysin dose. However, in some animals recolonization was noted within 48 h, which was caused most likely by bacteria previously internalized in epithelial cells of the mucous membrane. Importantly, isolated bacteria were sensitive to