Cover illustration: Schematic representation of the cell wall of Gram-negative bacteria.

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

de Groot, Ronald

Antibiotic resistance in *Haemophilus influenzae* / Ronald de Groot. - Rotterdam: Erasmus Universiteit, Afdeling Kindergeneeskunde. - [S.l. : s.n.]. - ill.
Thesis Rotterdam. - With ref. - With summary in Dutch.
ISBN 90-9004237-7
Subject heading: Antibiotic resistance in *Haemophilus influenzae.*

ANTIBIOTIC RESISTANCE IN HAEMOPHILUS INFLUENZAE

ANTIBIOTISCHE RESISTENTIE IN HAEMOPHILUS INFLUENZAE

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. C.J. Rijnvos
en volgens besluit van het College van Dekanen.
De openbare verdediging zal plaatsvinden op
woensdag 19 juni 1991 om 15:45 uur

door

Ronald de Groot

geboren te Rotterdam.

PROMOTIECOMMISSIE:

Promotoren:	Prof. Dr. H.J. Neijens
	Prof. Dr. A.L. Smith
Overige leden:	Prof. Dr. M.F. Michel
	Prof. Dr. W.P.M. Hoekstra

These studies were carried out at the University of Washington, Department of Pediatrics, Division of Pediatric Infectious Diseases, Seattle, U.S.A. and at the Erasmus University, Departments of Pediatrics and Microbiology, Rotterdam, The Netherlands.

The work was supported by grants from the Sophia Foundation for Medical Research, Rotterdam, The Netherlands.

Printing of this thesis was financially supported by Glaxo B.V., Nieuwegein, The Netherlands. Glaxo B.V. did the excellent job to make this book broadly available to all who are interested.

This thesis was printed by Haveka B.V. in Alblasserdam.

Aan Valerie, Steven, Mark, Arjan en Ewout
Aan mijn moeder

From quiet homes and first
beginning,
Out to the undiscovered ends,
There's nothing worth the wear of
winning,
But laughter and the love of
friends.

(Hilaire Belloc)

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

Haemophilus influenzae (*H. influenzae*) is a Gram-negative micro-organism responsible for a large variety of invasive and non-invasive diseases in developed and developing countries. *H. influenzae* is a major pathogen in childhood meningitis, bacteraemia, pneumonia, epiglottitis, septic arthritis, cellulitis, otitis media, sinusitis and other infections [3]. The major virulence determinant in *H. influenzae* is the production of capsular polysaccharide of type b [4, 5]. The recent development of conjugate vaccines, which contain a combination of polysaccharide capsule and carrier proteins such as diphtheria toxoid, tetanus toxoid, a variant of diphtheria toxin or outer membrane protein from meningococci has opened the perspective for future prevention of invasive *H. influenzae* diseases [2]. The development of a human vaccine against unencapsulated *H. influenzae*, which is a major cause of otitis media, sinusitis, pneumonia and chronic respiratory tract infections, is still in an experimental phase [1, 6]. It will certainly take many years before effective vaccines against non-typeable *H. influenzae* have been developed. Meanwhile an adequate antibiotic treatment of both invasive and non-invasive infections by *H. influenzae* remains of paramount importance in order to decrease the considerable morbidity and mortality.

In the early 1970s the treatment of choice for infections caused by *H. influenzae* was ampicillin. However, in the last two decades a significant increase has been noted in the prevalence of resistance in *H. influenzae* to ampicillin and many other antibiotics. Similar increases in the prevalence of antibiotic resistance have been documented in a still growing number of other micro-organisms. Resistance to antimicrobial agents has become a global problem with a major impact on the health care in developed and developing countries. Understanding of the epidemiology, the mechanisms of resistance and the molecular basis of antibiotic resistance may contribute to develop strategies intended to reduce or contain antibiotic resistance.

1.1 AIMS OF THE STUDIES

The studies presented in this thesis were undertaken to investigate the clinical importance, the dissemination, the biochemical mechanism(s) and the molecular basis of resistance to selected antibiotics in *H. influenzae*.

Chapter 2 gives a general view of the basis of antibiotic resistance in micro-organisms. The epidemiology, the molecular basis and the biochemical mechanisms of antibiotic resistance in bacteria are summarized. Subsequently, strategies which may result in a reduction of antibiotic resistance are discussed.

Chapter 3 describes the mode of action, the clinical indications and the currently documented mechanisms of resistance to trimethoprim.

Chapter 4 briefly describes the clinical importance and the different types of infection caused by encapsulated and unencapsulated *H. influenzae*. The problems with in vitro susceptibility testing are discussed in relation to the usefulness of specific antibiotics in the treatment of *H. influenzae* infections. Subsequently, the epidemiology, mechanisms of resistance and molecular basis of resistance to major antibiotics are reviewed. The consequences of the increase in antibiotic resistance for the therapy of invasive infections such as meningitis and non-invasive infections including otitis media will be discussed.

Chapters 5, 6 and 7 describe studies on the mechanism and genetic basis of resistance to trimethoprim in *H. influenzae*. The choice of trimethoprim was determined by the importance of this antibiotic in the treatment of infections caused by unencapsulated *H. influenzae*. At the onset of these studies the following questions were formulated:

1. Is it possible to perform accurate MIC determinations on thymidine free media to detect trimethoprim-resistant *H. influenzae*?

2. If trimethoprim-resistant isolates are detected, what is the level of resistance?

3. Is it possible to transfer resistance by means of conjugation or transformation and is the resistance marker chromosomally or plasmid-encoded?

4. Is it possible to clone the gene encoding trimethoprim resistance in a plasmid and transform this plasmid in a trimethoprim-susceptible *Escherichia coli* resulting in the expression of trimethoprim resistance?

5. What is the mechanism of resistance to trimethoprim in *H. influenzae*?

6. What is the molecular basis of resistance to trimethoprim in *H. influenzae*?

The questions 1 until 4 are addressed in chapter 5. The data presented in this chapter demonstrate, that trimethoprim-resistant *H. influenzae* strains were detected with MICs varying from 10 to > 200 μg/ml. Trimethoprim resistance could be transferred by conjugation and transformation. The mechanism of transfer is shown to be not plasmid-mediated, but chromosomally encoded. The gene encoding trimethoprim resistance in *H. influenzae* is cloned and the recombinant plasmid transduced and expressed in *E. coli*. By the use of an intragenic probe it is shown, that the gene encoding trimethoprim resistance in *H. influenzae* has no significant homology with trimethoprim resistance encoding genes in different Gram-negative micro-organisms. A preliminary characterization of the mechanism of trimethoprim resistance indicates, that this is caused by overproduction of dihydrofolate reductase in the trimethoprim-resistant strains.

Chapter 6 describes into more detail the mechanism of resistance to trimethoprim in *H. influenzae*. Dihydrofolate reductases from isogenic trimethoprim-resistant and -susceptible *H. influenzae* strains are highly purified by gel-filtration and dye-ligand chromatography. Characterization of the purified proteins by gel-electrophoresis, isoelectric focusing, Michaelis-Menten kinetics and peptide mapping indicates, that trimethoprim resistance in *H. influenzae* is a result from overproduction of structurally altered dihydrofolate reductase(s).

Chapter 7 describes further studies on the molecular basis of resistance to trimethoprim (see question 6 above). The gene encoding trimethoprim resistance is subcloned. A DNA fragment of approximately 1.6-kilobase, which still encodes resistance to trimethoprim, is sequenced. Analysis of the DNA sequence indicates the presence of an open reading frame (ORF) with 480 nucleotides. This gene encodes a protein with a predicted molecular mass of 17.760 kDa. The deduced amino acid sequence shows significant homology with several prokaryotic and eukaryotic DHFRs. The putative promoter sequences and ribosome binding site are very similar to those in *E. coli* clinical and laboratory strains, which overproduce dihydrofolate reductase. The nucleotide sequence 5' of the DHFR gene contains a second 561 basepair ORF on the complementary strand, which is transcribed in the opposite direction. The deduced protein has 62.9% identity with the N-terminal sequence of the *E. coli* proB gene encoding γ-glutamyl-kinase. This gene is preceded by promoter sequences and a potential ribosome binding site.

Chapter 8 describes the genetic relatedness of multiple antibiotic resistance determinants in *H. influenzae* isolates from different geographic regions in Spain. These isolates are resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, sulfamethoxazole, and trimethoprim. The mechanism of plasmid-mediated resistance to ampicillin, chloramphenicol and kanamycin is characterized. By the use of the intragenic probe mentioned in Chapter 5 it is shown, that resistance to trimethoprim in these strains is chromosomally encoded. Restriction endonuclease digestion of plasmid DNA from 10 multiple antibiotic-resistant *H. influenzae* strains was performed with a variety of restriction enzymes. Several restriction patterns could be distinguished. Southern blots of plasmid DNA using three antimicrobial resistance probes also revealed different patterns of hybridization. The results of this study indicate, that multiple antibiotic-resistant *H. influenzae* do not arise from a single clone. One may conclude, that both plasmid and chromosomal resistance evolved independently from several sources.

Chapter 9 summarizes the results of the studies presented in Chapters 5 until 8 and discusses the data in relation to those reported in the literature.

1.2 REFERENCES

1 **Brinton C.C., Carter M.J., Derber D.B., Kar S., Kramarik J.A., To A.C.C., To S.C.M., Wood S.W.** *Design and development of pilus vaccines for Haemophilus influenzae diseases.* Pediatr Infect Dis J 1989;8:S54-S61.

2 **Eskola J., Peltola H., Takala A.K., Käythy H., Hakulinen M., Karanko V., Kela E., Rekola P., Rönnberg P.R., Samuelson J.S., Gordon L.K., Mäkelä P.H.** *Efficacy of Haemophilus influenzae type b polysaccharide-diphtheria toxoid conjugate vaccine in infancy.* N Engl J Med 1987;317:717-722.

3 **Mendelman P.M., Smith A.L.** *Haemophilus influenzae.* In: Textbook of Pediatric Infectious Diseases, (Feigin R.D., Cherry R.D. eds.) pp 1142-1163, W.B. Saunders Company, 1987.

4 **Moxon E.R., Vaughn K.A.** *The type b capsular polysaccharide as a virulence determinant of Haemophilus influenzae: studies using clinical isolates and laboratory transformants.* J Infect Dis 1981;143:517-524.

5 **Moxon E.R., Deich R.A., Connelly C.** *Cloning of chromosomal DNA from Haemophilus influenzae: its use for studying the expression of type b capsule and virulence.* J Clin Invest 1984;73:298-306.

6 **Murphy T.F., Campagnari A.A., Nelson M.B., Apicella M.A.** *Somatic antigens of Haemophilus influenzae as vaccine components.* Pediatr Infect Dis J 1989;8:S66-S68.

CHAPTER 2

THE BASIS OF ANTIBIOTIC RESISTANCE IN MICRO-ORGANISMS

CHAPTER 2

THE BASIS OF ANTIBIOTIC RESISTANCE IN MICRO-ORGANISMS

2.1 THE EPIDEMIOLOGY OF ANTIMICROBIAL RESISTANCE

The emergence of resistance to commonly used antimicrobial agents has become a major problem in both developed and developing nations [7, 12, 21, 24]. Studies of the prevalence of antibiotic resistance in hospital micro-organisms have been complicated by methodological problems. These include the lack of a uniform definition of resistance, selection biases or insufficient control of confounding variables [20]. The increasing prevalence of antimicrobial resistance in hospital micro-organisms has been attributed to (1) changes in the relative frequency of "resistant bacteria" such as *Pseudomonas* or *Serratia*, (2) increased numbers of immunocompromised patients, (3) the introduction of new procedures and techniques such as invasive monitoring, (4) changes in inherent characteristics of micro-organisms and (5) variation in the practices of antibiotic usage [20, 21]. Some studies did not show uniform increases in the prevalence of resistance during the last decade [1]. However, the Task Force 2 of the NIH study on antibiotic use and antibiotic resistance worldwide documented an increase in resistance to "newer" antibiotics including gentamicin, methicillin and trimethoprim [24]. Resistance to trimethoprim, which is the major subject of chapters 3, 5, 6 and 7 in this thesis, has substantially increased in *Enterobacteriaceae*, *Salmonella* and *Shigella* isolates [24]. The clinical importance of the emergence of antibiotic-resistant micro-organisms may be illustrated by the fact, that infections by resistant bacteria are more likely to be associated with prolonged illness, frequent hospitalization, prolonged hospitalization and a higher death rate [12].

2.2 FACTORS ASSOCIATED WITH AN INCREASE IN ANTIMICROBIAL RESISTANCE

There is general agreement among experts, that the increase in antimicrobial resistance in hospital micro-organisms is related to the administration of antibiotics [29]. McGowan has published seven lines of evidence to support a linkage between hospital usage of antibiotics and the emergence of antimicrobial resistance (Table 2.1) [21]. However, the author also points to the fact, that this relation is not a universal phenomenon, but is applicable to specific drugs, micro-organisms and hospital environments [21]. The emergence of antibiotic-resistant hospital bacteria is determined by several additional factors including selection of resistant strains in treated individuals, the mechanism of transfer of resistance and the routes of transmission for bacteria and their resistance factors. The introduction of subtherapeutic amounts of antibiotics to animal feeds for the purpose of growth promotion has raised major concern because of the selection of multi-resistant micro-organisms [13, 26]. Holmberg et al. have shown, that the development of severe infections with drug-resistant *Salmonella newport* was associated with prior use of antibiotics, which constituted selective pressure for the growth of resistant micro-organisms [10]. Animal to man transmission of antibiotic-resistant *Salmonella* has been

Table 2.1 Evidence supporting a linkage between antibiotic resistance and use of antibiotics [21].

A.	Resistance is more prevalent in bacterial strains causing hospital infection, than in micro-organisms from community-acquired cases.
B.	During outbreaks patients with resistant strains are more likely to have received prior antibiotic therapy than are controls.
C.	Changes in the use of antibiotics lead to changes in prevalence of antibiotic resistance.
D.	Hospital areas such as intensive care units with highest usage of antibiotics have the highest prevalence of resistant bacteria.
E.	Increasing duration of exposure to antibiotics in the hospital is associated with increasing likelihood of colonization or infections with resistant micro-organisms.
F.	Increasing dosage of antibiotics leads to greater likelihood of superinfections or colonization with resistant micro-organisms.
G.	A biologic model can be proposed to explain the relationship between the proposed cause and effect.

identified in 38 outbreaks investigated by the Centers for Disease Control between 1971 and 1983 [11]. The case fatality rate for patients with multi-resistant *Salmonella* was 21 times higher than the case fatality rate associated with susceptible micro-organisms [11]. Bacteria such as *Escherichia coli* may also rapidly spread from an animal reservoir to the intestinal tract of humans and other animals in the absence of antibiotic selection [18]. Gastrointestinal colonization with these strains may persist for several months. The importance of transfer of antibiotic resistance genes from animal sources to humans may also be illustrated by the similarity of genetic determinants of resistance. An example is the homology between the genes encoding ROB-1 β-lactamase in *Haemophilus influenzae (H. influenzae)* strains (only found in humans) and in porcine *Haemophilus pleuropneumoniae* isolates and bovine and porcine *Pasteurella* strains [15].

2.3 MOLECULAR BASIS OF ANTIBIOTIC RESISTANCE

The six major genetic mechanisms of resistance to antibiotics are: (1) intrinsic resistance, (2) chromosomal mutation, (3) plasmid-mediated, (4) transposon-mediated, (5) integron-mediated and (6) tolerance.

Intrinsic resistance.

Intrinsic resistance indicates the natural resistance to antibiotics, that a majority of bacteria possess to certain antibiotics. This type of resistance may be caused by the presence of a permeability barrier for the antibiotic as has been shown in aminoglycoside-resistant anaerobes. Alternatively intrinsic resistance may be due to an insensitive target site or the induction of repressed genes in the presence of antibiotics. An example of this latter mechanism is the production of chromosomally encoded inducible β-lactamases in *Klebsiella*.

Chromosomal mutation

Chromosomal mutations are changes in the DNA sequence of bacterial chromosomes resulting in the synthesis of altered proteins or other molecules. Mutations may also lead to changes in the amount of protein formed. Mutations have been detected in a large variety of genes including those encoding penicillin-binding proteins, RNA polymerase, DNA gyrase, ribosomal proteins and bacterial energy generating systems. Mutations in these genes may lead to resistance to specific antibiotics. Often these mutations are associated with a decrease in virulence. Antibiotics commonly provide the selection pressure for generation of these mutants.

Plasmids

Plasmids are extrachromosomal circular pieces of DNA, that replicate independently of the host cell chromosome. Resistance plasmids are found in a wide variety of micro-organisms. They may be classified according to their capacity to coexist in the same host in a large number of incompatibility groups. The plasmid genome can encode a variety of proteins important in replication, fertility, resistance to antibiotics, toxic metals or bacteriophages, cell adhesion, virulence or other factors. Plasmid DNA may be rapidly transmitted between micro-organisms of the same species or different species by means of conjugation and transformation (see section 2.4).

Transposons

Transposons are linear DNA sequences ranging from 2.5 to 23-kb capable of transferring from one DNA molecule to another. Transposons differ from plasmids in two important ways: 1) they cannot replicate independently and must therefore be contained as part of a plasmid, bacteriophage of host chromosome and 2) transposons do not require extensive homology in order to insert into a target DNA sequence. Transposons always contain at their ends inverted or direct terminal repeats or short target site duplications. Some of these inverted sequences (insertion sequences) are capable of independent movement. The molecular mechanisms of transposon-mediated transfer of drug resistance genes and the implications for the spread of drug resistance genes throughout bacterial populations have been reviewed by Lupski [16].

Integrons

Recently a novel family of potentially mobile elements has been described within some bacterial chromosomes [28]. These elements called "integrons" lack inverted or direct terminal repeats at their ends, whereas their genome does not encode the proteins necessary for transposition. Integrons are characterized by the presence of site-specific integration functions. They are able to acquire various antibiotic resistance genes and express these genes by supplying the promoter [28]. Integron elements have been shown to encode trimethoprim-resistant dihydrofolate reductases and sulfonamide-resistant dihydropteroate synthetases [28].

Tolerance

Tolerance is defined as a larger than 32 fold ratio between the minimal bactericidal concentration (MBC) and the minimal inhibitory concentration (MIC). This type of resis-

tance is characterized by the inhibition of growth of micro-organisms in the absence of lysis. Tolerance is mainly detected in Gram-positive micro-organisms such as *Staphylococcus aureus* and may be due to a defect in autolysins. The clinical importance of this type of resistance is not yet well established.

2.4 GENETIC TRANSFER OF ANTIBIOTIC RESISTANCE

Four different ways of gene transfer from one micro-organism to another have been described. These are conjugation, transformation, transduction and transposition.

Conjugation

Conjugation is a process whereby DNA is transferred by cell to cell contact from a donor micro-organism to a recipient. Antibiotic resistance genes are frequently encoded by conjugative plasmids or self-transmissable transposons. Conjugative plasmids are widespread in Gram-positive and Gram-negative micro-organisms and may rapidly disseminate between different bacterial species. The mechanisms of plasmid-determined resistance to antimicrobial agents have been reviewed by Davies and Smith [6]. Experimental data on conjugation as a method of transferring trimethoprim resistance genes in *H. influenzae* are described in Chapter 5 of this thesis.

Transformation

Transformation is a process, whereby DNA from a donor micro-organism is taken up by a recipient and incorporated into its genome. Micro-organisms such as *H. influenzae* are only able to internalize homologous DNA, whereas other bacteria are also capable of transformation by heterologous DNA. Transformation is a very efficient mechanism of transfer of antibiotic resistance in *H. influenzae*. This is illustrated by experiments described in chapters 5 and 8.

Transduction

Transduction is the process whereby genes are transferred through bacteriophages. Bacteriophages are small viruses, which replicate as obligate intracellular parasites in bacteria. Donor and recipient micro-organisms need to express phage-specific cell surface receptors in order to be able to transfer bacteriophage DNA. Transduction plays an important role in the transmission of resistance in *Staphylococcus aureus* and *Streptococcus pyogenes*.

Transposition

Transposition is the mechanism by which transposons may transfer genes between conjugative and non-conjugative plasmids and chromosomes (see also section 2.3).

2.5 BIOCHEMICAL MECHANISMS OF ANTIBIOTIC RESISTANCE

The seven major mechanisms of antimicrobial resistance have been summarized in Table 2.2 and are discussed in more detail below.

Modification of the target site

Resistance of the antibiotic target site has been reviewed by Reynolds [25]. Penicillin-binding proteins (PBPs) catalyze reactions in the peptidoglycan cell wall biosynthesis. Chromosomal mutations in genes encoding PBPs are described in an increasing number of micro-organisms including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *H. influenzae* and *Neisseria gonorrhoeae*. These mutations may result in resistance to a variety of β-lactam antibiotics.

DNA gyrase is involved in the introduction of negative supercoils into closed circular DNA and is an important enzyme involved in DNA replication. Mutations in DNA gyrase may lead to resistance to novobiocin or quinolones.

RNA polymerase, the target enzyme of rifampin, is involved in the transcription of DNA to RNA. Mutations in this enzyme may result in resistance to rifampin.

Bacterial ribosomes consist of 2 subunits each containing RNA and proteins. The 30S subunit contains 21 proteins and 16S RNA, whereas the 50S subunit contains 32 proteins and 23S and 5S RNA. Mutations in ribosomal RNA may result in resistance to aminoglycosides. Plasmid-mediated production of enzymes, which methylate the 50S ribosomal subunit may be responsible for the decrease in affinity of ribosomes for macrolide antibiotics.

Decreased drug accumulation

Antibiotic resistance resulting from decreased drug accumulation has been reviewed by Chopra [5]. This type of resistance may be intrinsic or acquired. Examples of intrinsic resistance are the relative inability of hydrophobic antibiotics to diffuse through the porins of the outer membrane of Gram-negative micro-organisms [22, 23]. Gram-negative micro-

Table 2.2 Mechanisms of antibiotic resistance in micro-organisms.

A.	Modification of the target site	
	- penicillin-binding proteins	
	- DNA gyrase	
	- RNA polymerase	
	- ribosome	
B.	Decreased drug accumulation	
	- intrinsic resistance	
	- acquired resistance:	chromosomally mediated
		plasmid-mediated
C.	Antibiotic inactivation	
	- β-lactamases	
	- chloramphenicol acetyltransferases	
	- aminoglycoside modifying enzymes	
D.	Metabolic bypass	
	- altered dihydrofolate reductases	
	- altered dihydropteroate synthetases	
E.	Increasing the level of enzyme inhibited by the antibiotic	
	- gene amplification	
	- mutation in regulatory gene	
F.	Tolerance	
	- defect in autolysins	
G.	Decreasing metabolic requirement for the pathway inhibited by the antibiotic	
	- thymine auxotrophy	

organisms such as *Pseudomonas aeruginosa* have a markedly reduced permeability of the outer membrane for hydrophillic antibiotics. This is caused by differences in porin characteristics compared with other Gram-negative micro-organisms such as *Escherichia coli* [9, 14]. Acquisition of resistance may be caused by chromosomal mutations or plasmid-mediated mechanisms. Plasmid-located genes may encode specific membrane-located proteins (called Tet A, B and C), which promote energy-dependent efflux of tetracycline. Tetracycline normally inhibits the bacterial protein synthesis by interaction with the 30S ribosomal subunit. Chromosomal mutations in carrier proteins, porins or membrane products may also result in decreased permeability of the bacterial cell wall.

This mechanism has been reported for a variety of antibiotics including aminoglycosides, β-lactam antibiotics, chloramphenicol, tetracycline, quinolones and others.

Antibiotic inactivation

A third important mechanism of resistance is the inactivation of antibiotics by enzymes. Three major groups of enzymes have been described: β-lactamases, chloramphenicol acetyltransferases and aminoglycoside inactivating enzymes. β-lactamases hydrolyse the β-lactam ring of β-lactam antibiotics thereby destroying their antibacterial activity. Different schemes have been developed to classify the wide variety of β-lactamases. These classifications are based on differences in isoelectric points, substrate profiles, inhibitor profiles, plasmid or chromosomal location of the genes, immunologic reactivity, amino acid sequences or molecular structure [4]. In addition the production of β-lactamases may be at a constant rate (constitutive) or induced by the presence of substrate (inducible). It has been suggested, that β-lactamases and penicillin-binding proteins have a common, but distant, evolutionary origin [27]. Plasmid- or transposon-specified β-lactamases are found in a wide variety of Gram-positive and Gram-negative micro-organisms including *H. influenzae* (see Chapters 4 and 8 of this thesis).
Chloramphenicol acetyltransferase (CAT) is a plasmid- or transposon-mediated enzyme, which catalyzes the diacetylation of chloramphenicol with acetyl coenzyme A. The resulting compound is not able to inhibit bacterial protein synthesis. CAT produced by staphylococci is inducible, whereas CAT produced by Gram-negative bacteria is constitutive. CAT-mediated resistance to chloramphenicol is a major problem in many Gram-negative micro-organisms including *H. influenzae* (see chapters 4 and 8).
Aminoglycoside-modifying enzymes are divided into three groups: acetyltransferases, adenyltransferases and phosphotransferases. Aminoglycosides normally bind to the 30S and 50S subunits of ribosomes thereby causing translational misreading and inhibition of elongation of protein chains. Resistance by aminoglycoside-modifying enzymes is caused by an interplay between the rate of inactivation of aminoglycosides and the rate of drug transport by the aminoglycoside-transport system in bacteria [2]. Aminoglycoside-inactivating enzymes are the major cause of aminoglycoside resistance in a large variety of Gram-negative micro-organisms including *H. influenzae* (see also chapters 4 and 8).

Metabolic bypass

Metabolic bypass is the mechanism of resistance characterized by the synthesis of an alternative target site. This mechanism is the principal cause of resistance to sulfonamides and trimethoprim. In most cases the enzymes are plasmid-encoded. Sulfonamide resistance is caused by the production of an altered dihydropteroate synthetase. Trimethoprim resistance will be discussed in further detail in Chapter 3.

Tolerance

Tolerance as a mechanism of resistance has been described in section 2.3.

Increased enzyme production

An increase in the level of enzymes inhibited by an antimicrobial agent may also lead to resistance. This mechanism is important in resistance to trimethoprim and will be described in further detail in Chapters 3, 5, 6 and 7.

Decreased metabolic requirement

A decrease in the metabolic requirement for the pathway inhibited by a drug may also lead to resistance. This mechanism has been studied in thymidine requiring mutants of *H. influenzae*, which do not utilize the tetrahydrofolate pathway [19].

2.6 STRATEGIES FOR REDUCTION OF ANTIBIOTIC RESISTANCE

Antimicrobial resistance may be subdivided into two major categories termed "positive function resistance" and "persistence" [3]. This classification has important consequences for the strategies, which may be chosen to contain antimicrobial resistance or control an increase in resistance.

Positive function resistance is the mechanism, whereby an antibiotic acquires additional gene function(s), which regulate resistance to one or more antibiotics. This type of resistance generally results from the transfer of plasmid or transposon DNA into a bacterial strain. The control of this type of resistance may be achieved either by infection control measures or by the development of new antimicrobial compounds, which are insensitive to or overcome antibiotic resistance. Positive function resistance usually is present at the onset of therapy and rarely develops during treatment with antibiotics. Overall control of the dissemination of this type of resistance needs to be focused on a population basis.

Persistence is a mechanism resulting from mutations in structural or regulatory genes or from phenotypic changes. This type of resistance only develops during antibiotic treatment. The genotypic or phenotypic changes leading to antibiotic resistance often result in deleterious effects on the bacterial metabolism. Once the antibiotic selection pressure is removed, micro-organisms will commonly revert to their original phenotype or

genotype. Mechanisms of persistence include changes in outer membrane permeability, penicillin-binding proteins, mutations in bacterial target enzymes such as DNA gyrase or RNA polymerase. Strategies to prevent persistence include the use of individually designed drug combinations or rotating drugs with different targets or routes of cellular entry [3]. Persistence may develop as a complication of antibiotic treatment with any type of antibiotic. The administration of combinations of antibiotics has been proven to prevent this class of resistance in *Myobacterium tuberculosis*. Some animal studies also indicate, that combination therapy may limit the emergence of resistance [8]. However, human data currently do not support this practice [29].

The emergence of resistant hospital micro-organisms may be attributed to both "persistence" and "positive function resistance" mechanisms. There is wide agreement among experts, that resistance may be prevented by a more prudent use of antibiotics [20, 29]. The Infectious Diseases Society of America has published guidelines for improving the use of antibiotics, and has made suggestions to implement these guidelines [17]. The effects of implementation of these guidelines should be evaluated in future prospective studies. In addition a more restrictive application of antibiotics in veterinary use of antibiotics may reduce the selection and subsequent spread to humans of antibiotic-resistant micro-organisms [26].

2.7 REFERENCES

1 **Barry A.L., Jones R.N.** *Bacterial antibiotic resistance before and after clinical application in the United States.* Bull N Y Acad Med 1987;63:217-230.

2 **Bryan L.E.** *General mechanisms of resistance to antibiotics.* J Antimicrob Chemother 1988;22:S1-S15.

3 **Bryan L.E.** *Two forms of antimicrobial resistance: bacterial persistence and positive function resistance.* J Antimicrob Chemother 1989;23:817-823.

4 **Bush K.** *Recent developments in β-lactamase research and their implications for the future.* Rev Infect Dis 1988;10:681-690.

5 **Chopra I.** *Antibiotic resistance resulting from decreased drug accumulation.* Brit Med Bull 1984;40:11-17.

6 **Davies J., Smith D.I.** *Plasmid-determined resistance to antimicrobial agents.* Ann Rev Microbiol 1978;32:469-518.

7 **Farrar W.E.** *Antibiotic resistance in developing countries.* J Infect Dis 1985;152:1103-1106.

8 **Hamzehpour M.M., Pechère J.C., Marchou B., Auckenthaler R.** *Combination therapy: a way to limit emergence of resistance.* Am J Med 1986;80:S138-S142.

9 **Hancock R.E.W., Woodruff W.A.** *Roles of porin and β-lactamase in β-lactam resistance of Pseudomonas aeruginosa.* Rev Infect Dis 1988;10:770-781.

10 **Holmberg S.D., Osterholm M.T., Senger K.A., Cohen M.L.** *Drug-resistant Salmonella from animals fed antimicrobials.* N Engl J Med 1984;311:617-622.

11 **Holmberg S.D., Wells J.G., Cohen M.L.** *Animal-to-man transmission of antimicrobial-resistant Salmonella: investigations of U.S. outbreaks, 1971-1983.* Science 1984;225:833-835.

12 **Holmberg S.D., Solomon S.L., Blake P.A.** *Health and economic impacts of antimicrobial resistance.* Rev Infect Dis 1987;9:1065-1078.

13 **Levy S.B.** *Man, animals and antibiotic resistance.* Pediatr Infect Dis J 1985;4:3-5.

14 **Livermore D.M.** *Permeation of β-lactam antibiotics into Escherichia coli, Pseudomonas aeruginosa, and other Gram-negative bacteria.* Rev Infect Dis 1988;10:691-698.

15 **Livrelli V.O., Darfeuille-Richaud A., Rich C.D., Joly B.H., Martel J.L.** *Genetic determinant of the ROB-1 β-lactamase in bovine and porcine Pasteurella strains.* Antimicrob Agents Chemother 1988;32:1282-1284.

16 **Lupski J.R.** *Molecular mechanisms for transposition of drug-resistance genes and other movable genetic elements.* Rev Infect Dis 1987;9:357-368.

17 **Marr J.J., Moffet H.L., Kunin C.M.** *Guidelines for improving the use of antimicrobial agents in hospitals: a statement by the Infectious Diseases Society of America.* J Infect Dis 1988;157:869-876.

18 **Marshall B., Petrowski D., Levy S.B.** *Inter- and intraspecies spread of Escherichia coli in a farm environment in the absence of antibiotic usage.* Proc Natl Acad Sci USA 1990;87:6609-6613.

19 **Maskell R., Okubadejo O.A., Payne R.H.** *Thymine-requiring bacteria associated with co-trimoxazole therapy.* Lancet 1976;I:834.

20 **McGowan J.E.** *Antimicrobial resistance in hospital organisms and its relation to antibiotic use.* Rev Infect Dis 1983;5:1033-1048.

21 **McGowan J.E.** *Is antimicrobial resistance in hospital micro-organisms related to antibiotic use?* Bull N Y Acad Med 1987;63:253-268.

22 **Nikaido H., Vaara M.** *Molecular basis of bacterial outer membrane permeability.* Microbiol Rev 1985;49:1-32.

23 **Nikaido H.** *Bacterial resistance to antibiotics as a function of outer membrane permeability.* J Antimicrob Chemother 1988;22:S17-S22.

24 **O'Brien T.F., the Members of Task Force 2.** *Resistance of bacteria to antibacterial agents:report of Task Force 2.* Rev Infect Dis 1987;9:S244-S260.

25 **Reynolds P.E.** *Resistance of the antibiotic target site.* Brit Med Bull 1984;40:3-10.

26 **Rijksinstituut voor Volksgezondheid en Milieuhygiëne.** *Veterinair antibioticagebruik en volksgezondheid.* Workshop 22 maart 1990, (van Klingeren B., Nieuwenhuys J.H.M. eds.) Bilthoven, The Netherlands.

27 **Spratt B.G., Cromie K.D.** *Penicillin-binding proteins of Gram-negative bacteria.* Rev Infect Dis 1988;10:699-711.

28 **Stokes H.W., Hall R.M.** *A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons.* Mol Microbiol 1989;3:1669-1683.

29 **Young L.S.** *Antimicrobial resistance: implications for antibiotic use.* Am J Med 1986;80:S35-S39.

CHAPTER 3

TRIMETHOPRIM: MODE OF ACTION, CLINICAL USE AND MECHANISMS OF RESISTANCE

CHAPTER 3

TRIMETHOPRIM: MODE OF ACTION, CLINICAL USE AND MECHANISMS OF RESISTANCE

3.1 INTRODUCTION

Trimethoprim, 2,4-diamino-5-(3',4',5'-trimethoxybenzyl)-pyrimidine is a synthetic compound with antibacterial and antiparasitic activity. The chemical structure of trimethoprim (Tmp) is shown in Figure 3.1. Trimethoprim is often used in combination with sulfamethoxazole (Tmp-Smx or co-trimoxazole) in the treatment of a wide variety of aerobic Gram-positive or Gram-negative infections and in the treatment of infections caused by *Pneumocystis carinii*. Additional indications for the use of co-trimoxazole have been reviewed by Kucers and Bennet [30].

3.2 MODE OF ACTION OF TRIMETHOPRIM

The mechanism of action of Tmp is by competitive binding to bacterial dihydrofolate reductase (DHFR) [8, 27]. The result of inhibition of DHFR activity is a decrease in the synthesis of tetrahydrofolate (TH_4) (see Figure 3.2). The selective activity of Tmp on prokaryotic cells is based on a several thousand-fold higher affinity for bacterial DHFRs, than for mammalian DHFRs. Another difference between the mammalian and eukaryotic

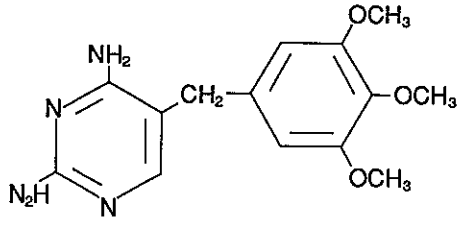

Figure 3.1 The structure of Trimethoprim [2,4-diamino-5-(3',4',5'-trimethoxylbenzyl)-pyrimidine].

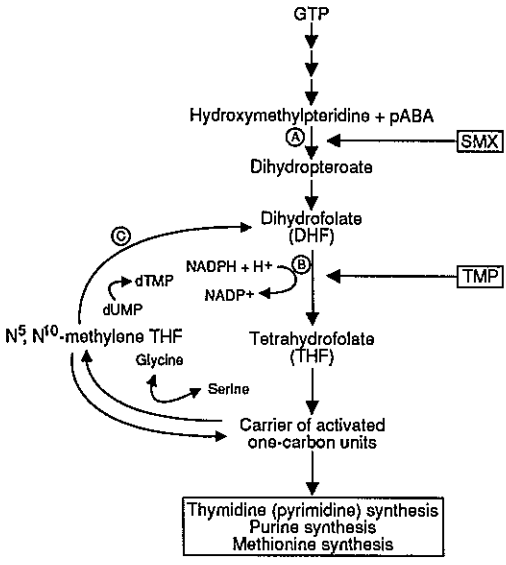

Figure 3.2 Pathway for the biosynthesis of tetrahydrofolate and for the interference of sulphamethoxazole (SMX) and trimethoprim (TMP).

Abbreviations: GTP, guanosine triphosphate; pABA, p-aminobenzoic acid;
A, dihydropteroate synthetase;
B, dihydrofolate reductase;
C, thymidilate synthetase.

folate metabolism is the inability of eukaryotes to synthesize the pteridine ring of the folic acid molecule (see Figure 3.3). Folate is hence obtained from diet or from micro-organisms in the intestinal tract. The activity of Tmp on bacterial DHFR leads to decreased production of TH_4 and has subsequently deleterious effects on the biosynthesis of purines, pyrimidines, methionine and serine (see Figure 3.2). This is a result of the important role of TH_4 at different oxidation levels as a carrier of one-carbon units (Figure 3.2). TH_4 also serves as an acceptor of one-carbon units in degradative reactions including the conversion of serine in glycine. 5,10-Methylene TH_4 may be oxidized to DH_2. This reaction is catalyzed by the enzyme thymidilate synthetase and involves the methylation of deoxyuridilate (dUMP) to deoxythymidylate (dTMP) (Figure 3.2). Deoxythymidylate is an important precursor for the synthesis of DNA and depletion of this

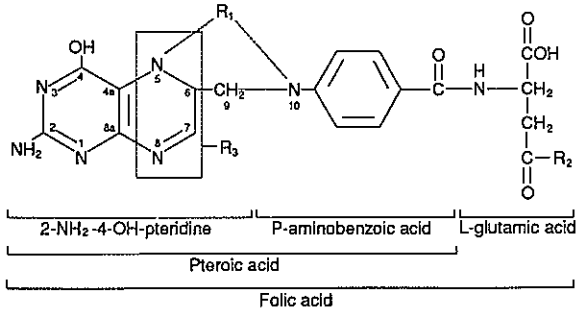

Figure 3.3 The structure of folic acid.

R1 indicates the substitution site for 1-carbon groups;
R2 indicates the site for polyglutamation;
R3 indicates the positions 5, 6, 7 and 8 of reduction of the pyrazine ring of the pteridine moiety.

compound will lead to cell death. Maskell et al. have described *Escherichia coli* strains and other bacteria with a mutation in the thymidilate synthetase [34]. These strains are highly resistant to Tmp. They can not longer synthesize thymidilate from folate and do not survive in the absence of thymine or thymidine in media. The folate biosynthesis may also be inhibited by sulfonamides such as sulfamethoxazole (Smx). Smx competitively inhibits the enzyme dihydropteroate synthetase which is responsible for the conversion of hydroxymethylpteridine to dihydropteroate (Figure 3.2). The synergistic activity of Tmp and Smx may be a result from the cyclic configuration of the folate pathway [24].

3.3 CLINICAL USE OF TRIMETHOPRIM OR CO-TRIMOXAZOLE IN CHILDREN

Co-trimoxazole is a first choice agent in the treatment of urinary tract infections, respiratory tract infections, otitis media, infections with *Salmonella* and *Shigella spp.*, *Vibrio cholerae*, enterotoxigenic *E. coli*, *Nocardia sp.* and *Pneumocystis carinii* [30]. Tmp monotherapy has been used with good therapeutic effect in the treatment of urinary tract infections, respiratory tract infections and infections with *Salmonella* and in the chemoprophylaxis against *Pneumocystis carinii* [30]. The indications for use of Tmp in the treatment of infections caused by *H. influenzae* are discussed in chapter 4.

3.4 MECHANISMS AND EPIDEMIOLOGY OF BACTERIAL RESISTANCE TO TRIMETHOPRIM

Trimethoprim-resistant micro-organisms have been reported since shortly after the introduction of Tmp in the 1960s. The different mechanisms of resistance have been reviewed in several publications [3, 9, 29, 48, 49] and are summarized in Table 3.1. The production of trimethoprim-resistant plasmid-encoded DHFRs has become the most important mechanism of resistance to Tmp. So far 7 major classes and five further subgroups of Tmp-resistant DHFRs have been described in Gram-negative micro-organisms [3]. In Gram-positive bacteria one major class has been described [4]. Classification of DHFRs is based on a large variety of techniques. These include isoelectric point determination, amino acid analysis, sequence analysis, molecular mass determination, MIC determination, pH profile, Km values for dihydrofolate and NADPH, 50% inhibitory concentration (IC_{50}) of Tmp and plasmid or chromosomal location of the altered gene. The study of the molecular epidemiology of Tmp resistance has become a major tool to detect the evolution and dissemination of different Tmp-resistant DHFRs on a global scale [3, 18]. The overall level of plasmid-mediated Tmp resistance in *Enterobacteriaceae* (especially *E. coli* isolates from urinary tract infections) has steadily increased

Table 3.1 Mechanisms of resistance to trimethoprim[a].

Genetic basis	Mechanisms	Micro-organisms	References
Intrinsic resistance	Decreased cell wall permeability	*P. aeruginosa*	[52]
	Production of Tmp-resistant DHFR	*Neisseria, Nocardia, anaerobes*	[47, 48]
Chromosomal mutation	Decreased cell wall permeability	*Klebsiella, Enterobacter, Serratia*	[19, 22]
	Thymine auxotrophy	*E. coli, Proteus S. faecalis*	[33]
	Overproduction of DHFR	*S. pneumoniae, E. coli H. influenzae, S. aureus*	[16, 21, 32, 39, 41]
	Production of Tmp-resistant DHFR	*E. coli*	[42]
Plasmid- or transposon-mediated	Production of Tmp-resistant DHFR	*S. aureus, S. epidermidis, Enterobacteriaceae, Salmonella, Shigella, Vibro cholerae, P. cepacea*	[3, 4, 5, 10, 14, 17, 26, 46, 55, 56]
Integron-mediated	Production of Tmp-resistant DHFR	*E. coli*	[43, 44, 45]

[a] Abbreviations: DHFR, dihydrofolate reductase; Tmp, trimethoprim.

during the 1970s and 1980s from less than 10% to 30-40% in many European countries with the exception of the United Kingdom [18, 25, 28]. The increase in Tmp resistance is related to an increase in the consumption of Tmp resulting in a pronounced selection pressure [3, 28, 40, 50]. The level of Tmp resistance is below 10% in the United Kingdom and the United States [18]. It is yet unknown if this is a result of decreased selection pressure. Trimethoprim resistance has become a major problem in developing countries. The low price, the easy availability and the high consumption of Tmp in developing countries certainly contribute to the epidemic spread of Tmp resistance [2]. The Tmp resistance rate (strains with MIC > 1000 μg/ml) in urinary tract pathogens in Nigeria and

India is approximately 55% [31, 54]. A high rate of resistance in *Enterobacteriaceae* has also been reported in a study from South-Africa [53]. The selection and subsequent emergence of high level Tmp resistance in fecal *Escherichia coli* isolates from travellers to Mexico, who received daily profylaxis with Tmp or trimethoprim-sulfamethoxazole additionally supports the strong association between the use of Tmp and development of resistance [35]. Plasmid-mediated resistance of *Shigella* has become a major problem both in developed and developing countries [1, 7, 15, 20, 23]. Study of the evolution of plasmid-mediated resistance genes encoding Tmp-resistant DHFRs has indicated, that some of these plasmids may have spread from an animal reservoir (pigs) to humans [11, 12].

Chromosomal mutations to Tmp resistance are clinically less important, than plasmid-mediated resistance mechanisms. Nevertheless a variety of mechanisms has been described (Table 3.1). The regulatory mechanisms responsible for overproduction of DHFR or production of altered DHFRs have also been studied by in vitro mutagenesis. Altered DHFR molecules have been generated by in vitro mutations in *E. coli* and *S. pneumoniae* [6, 13, 36, 37, 38, 51]. Overproduction of DHFR caused by promoter mutations has been described in clinical isolates of *E. coli* [16]. The in vitro mutagenesis techniques have greatly contributed toward our understanding of the structure and function of dihydrofolate reductases.

3.5 REFERENCES

1 **Agodi A., Jones C., Threlfall E.J., d'Angelo M., Marranzano M.** *Molecular characterization of trimethoprim resistance in Shigella sonnei in Sicily.* Epidemiol Infect 1990;105:29-40.

2 **Amyes S.G.B., Young H.K.** *The genetics of bacterial trimethoprim resistance in tropical areas.* Trans R Soc Trop Med Hyg 1987;81:504-507.

3 **Amyes S.G.B., Towner K.J.** *Trimethoprim resistance; epidemiology and molecular aspects.* J Med Microbiol 1990;31:1-19.

4 **Archer G.L., Coughter J.P., Johnston J.L.** *Plasmid-encoded trimethoprim resistance in staphylococci.* Antimicrob Agents Chemother 1986;29:733-740.

5 **Barg N.L., Hutson F.S., Wheeler L.A., Thomson C.J., Amyes S.G.B., Wharton M., Schaffner W.** *Novel dihydrofolate reductases isolated from epidemic strains of trimethoprim/sulfamethoxazole-resistant Shigella sonnei.* J Infect Dis 1990;162:466-473.

6 **Benkovic S.J., Fierke C.A., Naylor A.M.** *Insights into enzyme function from studies on mutants of dihydrofolate reductase.* Science 1988;239:1105-1110.

7 **Bratoeva M.P., John J.F.** *Dissemination of trimethoprim-resistant clones of Shigella sonnei in Bulgaria.* J Infect Dis 1989;159:648-653.

8 **Burchall J.J.** *Mechanism of action of trimethoprim-sulfamethoxazole-II.* J Infect Dis 1973;128:S437-S441.

9 **Burchall J.J., Elwell L.P., Fling M.E.** *Molecular mechanisms of resistance to trimethoprim.* Rev Infect Dis 1982;4:246-254.

10 **Burns J.L., Llen D.M., Hedin L.A.** *Isolation and characterization of dihydrofolate reductase from trimethoprim-susceptible and trimethoprim-resistant Pseudomonas cepacea.* Antimicrob Agents Chemother 1989;33:1247-1251.

11 **Campbell I.G., Mee B.J., Nikoletti S.M.** *Evolution and spread of IncFIV plasmids conferring resistance to trimethoprim.* Antimicrob Agents Chemother 1986;29:807-813.

12 **Campbell I.G., Mee B.J.** *Mapping of trimethoprim resistance genes from epidemiologically related plasmids.* Antimicrob Agents Chemother 1987;31:1440-1441.

13 **Chen J.T., Mayer R.J., Flerke C.A., Benkovic S.J.** *Site-specific mutagenesis of dihydrofolate reductase from Escherichia coli.* J Cell Biochem 1985;29:73-82.

14 **Coughter J.P., Johnston J.L., Archer G.L.** *Characterization of a staphylococcal trimethoprim resistance gene and its product.* Antimicrob Agents Chemother 1987;31:1027-1032.

15 **Delgado R., Otero J.R.** *High-level resistance to trimethoprim in Shigella sonnei associated with plasmid-encoded dihydrofolate reductase type I.* Antimicrob Agents Chemother 1988;32:1598-1599.

16 **Flensburg J., Sköld O.** *Massive overproduction of dihydrofolate reductase in bacteria as a response to the use of trimethoprim.* Eur J Biochem 1987;162:473-476.

17 **Goldstein F.W., Chumpitaz J.C., Guevara J.M., Papadopoulou B, Acar J.F., Vieu J.F.** *Plasmid-mediated resistance to multiple antibiotics in Salmonella typhi.* J Infect Dis 1986;153:261-266.

18 **Goldstein F.W., Papadopoulou B., Acar J.F.** *The changing pattern of trimethoprim resistance in Paris, with a review of worldwide experience.* Rev Infect Dis 1986;8:725-737.

19 **Grey D., Hamilton-Miller J.M.T., Brumfitt W.** *Incidence and mechanisms of resistance to trimethoprim in clinically isolated Gram-negative bacteria.* Chemotherapy 1979;25:147-156.

20 **Griffin P.M., Tauxe R.V., Redd S.C., Puhr N.D., Hargrett-Bean N., Blake P.A.** *Emergence of highly trimethoprim-sulfamethoxazole-resistant Shigella in a native American population: an epidemiologic study.* Am J Epidem 1989;129:1042-1051.

21 **de Groot R., Chaffin D.O., Kuehn M., Smith A.L.** *Trimethoprim resistance in Haemophilus influenzae is due to altered dihydrofolate reductase(s).* Biochem J 1991;274:657-662.

22 **Gutmann L., Williamson R., Moreau N., Kitzis M.D., Collatz E., Acar J.F., Goldstein F.W.** *Cross-resistance to nalidixic acid, trimethoprim and chloramphenicol associated with alterations in outer membrane proteins of Klebsiella, Enterobacter and Serratia.* J Infect Dis 1985;151:501-507.

23 **Haider K., Huq M.I., Samadi A.R., Ahmad K.** *Plasmid characterization of Shigella spp. isolated from children with shigellosis and asymptomatic excretors.* J Antimicrob Chemother 1985;16:691-698.

24 **Harvey R.J.** *Synergism in the folate pathway.* Rev Infect Dis 1982;4:255-260.

25 **Heikkilä E., Renkonen O.V., Sunila R., Uurasmaa P., Huovinen P.** *The emergence and mechanisms of trimethoprim resistance in Escherichia coli isolated from outpatients in Finland.* J Antimicrob Chemother 1990;25:275-283.

26 **Heikkilä E., Siitonen A., Jahkola M., Fling M., Sundström L., Huovinen P.** *Increase of trimethoprim resistance among Shigella species, 1975-1988: analysis of resistance mechanisms.* J Infect Dis 1990;161:1242-1248.

27 **Hitchings G.H.** *Mechanism of action of trimethoprim-sulfamethoxazole-I.* J Infect Dis 1973;128:S433-S436.

28 **Huovinen P., Pulkkinen L., Helin H.L., Mäkilä M., Toivanen P.** *Emergence of trimethoprim resistance in relation to drug consumption in a Finnish hospital from 1971 through 1984.* Antimicrob Agents Chemother 1986;29:73-76.

29 **Huovinen P.** *Trimethoprim resistance.* Antimicrob Agents Chemother 1987;31:1451-1456.

30 **Kucers A., Bennett N.McK.** *Trimethoprim, Co-trimoxazole (Co-T), and other trimethoprim combinations.* In: Kucers A., Bennett N. eds. The use of Antibiotics. London: William Heinemann Medical Books, 1987;1118-1202.

31 **Lamikanra A., Ndep R.B.** *Trimethoprim resistance in urinary tract pathogens in two Nigerian hospitals.* J Antimicrob Chemother 1989;23:151-154.

32 **Lyon B.R., Skurray R.A.** *Antimicrobial resistance of Staphylococcus aureus: genetic basis.* Microbiol Rev 1987;51:88-134.

33 **Maskell R., Okubadejo O.A., Payne R.H.** *Thymine-requiring bacteria associated with co-trimoxazole therapy.* Lancet 1976;I:834.

34 **Maskell R., Okubadejo O.A., Payne R.H., Pead L.** *Human infections with thymine-requiring bacteria.* J Med Microbiol 1977;11:33-45.

35 **Murray B.E., Rensimer E.R., DuPont H.L.** *Emergence of high-level trimethoprim resistance in fecal Escherichia coli during oral administration of trimethoprim or trimethoprim-sulfamethoxazole.* N Engl J Med 1982;306:130-135.

36 **Sheldon R., Brenner S.** *Regulatory mutants of dihydrofolate reductase in Escherichia coli K12.* Mol Gen Genet 1976;147:91-97.

37 **Sheldon R.** *Altered dihydrofolate reductase in fol regulatory mutants of Escherichia coli K12.* Mol Gen Genet 1977;151:215-219.

38 **Sirotnak F.M., Hachtel S.L., Williams W.A.** *Increased dihydrofolate reductase synthesis in Diplococcus pneumoniae following translatable alteration of the structural gene. II. Individual and dual effects on the properties and rate of synthesis of the enzyme.* Genetics 1969;61:313-326.

39 **Sirotnak F.M.** *Increased dihydrofolate reductase synthesis in Diplococcus pneumoniae following translatable alteration of the structural gene. III. Further evidence on the extent of gene involvement.* Genetics 1970;65:391-406.

40 **Sköld O., Boethius G., Steen R.** *Correlation of drug utilization data for trimethoprim in a defined population with patterns of resistance among bacteria causing urinary tract infections.* Scand J Infect Dis 1986;18:451-455.

41 **Smith D.R., Calvo J.M.** *Nucleotide sequence of dihydrofolate reductase genes from trimethoprim-resistant mutants of Escherichia coli.* Mol Gen Genet 1982;187:72-78.

42 **Smith D.R., Rood J.I., Bird P.I., Sneddon M.K., Calvo J.M., Morrison J.F.** *Amplification and modification of dihydrofolate reductase in Escherichia coli.* J Biol Chem 1982;257:9043-9048.

43 **Stokes H.W., Hall R.M.** *A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons.* Mol Microbiol 1989;3:1669-1683.

44 **Sundström L., Rådström P., Swedberg G., Sköld O.** *Site-specific recombination promotes linkage between trimethoprim and sulfonamide resistance genes. Sequence characterization of dhfrV and sul1 and a recombination active locus of Tn21.* Mol Gen Genet 1988;213:191-201.

45 **Sundström L., Sköld O.** *The dhfr1 trimethoprim resistance gene of Tn7 can be found at specific sites in other genetic surroundings.* Antimicrob Agents Chemother 1990;34:642-650.

46 **Tabtieng R., Wattanasri S., Echeverria P., Seriwatana J., Bodhidatta L., Chatkaeomorakot A., Rowe B.** *An epidemic of Vibrio cholerae el tor inaba resistant to several antibiotics with a conjugative group c plasmid coding for type II dihydrofolate reductase in Thailand.* Am J Trop Med Hyg 1989;41:680-686.

47 **Then R.L., Angehrn P.** *Low trimethoprim susceptibility of anaerobic bacteria due to insensitive dihydrofolate reductases.* Antimicrob Agents Chemother 1979;15:1-6.

48 **Then R.L., Hermann F.** *Mechanisms of trimethoprim resistance in Enterobacteria isolated in Finland.* Chemotherapy 1981;27:192-199.

49 **Then R.L.** *Mechanisms of resistance to trimethoprim, the sulfonamides and trimethoprim-sulfamethoxazole.* Rev Infect Dis 1982;4:261-269.

50 **Towner K.J., Slack R.C.B.** *Effect of changing selection pressures on trimethoprim resistance in Enterobacteriaceae.* Eur J Clin Microbiol Infect Dis 1986;5:502-506.

51 **Villafranca J.E., Howell E.E., Voet D.H., Strobel M.S., Ogden R.C., Abelson J.N., Kraut J.** *Directed mutagenesis of dihydrofolate reductase.* Science 1983;222:782-788.

52 **Werner R.G., Goeth H.** *Trimethoprim, failure to penetrate into Pseudomonas aeruginosa cells.* FEMS Microbiol Lett 1984;23:201-204.

53 **Wylie B.A., Koornhof H.J.** *Trimethoprim resistance in Gram-negative bacteria isolated in South Africa.* J Antimicrob Chemother 1989;24:973-982.

54 **Young H.K., Jesudason M.V., Koshi G., Amyes S.G.B.** *Trimethoprim resistance amongst urinary pathogens in South India.* J Antimicrob Chemother 1986;17:615-621.

55 **Young H.K., Amyes S.G.B.** *Plasmid trimethoprim resistance in Vibrio cholerae: migration of the type I dihydrofolate reductase gene out of the Enterobacteriaceae.* J Antimicrob Chemother 1986;17:697-703.

56 **Young H.K., Skurray R.A., Amyes S.G.B.** *Plasmid-mediated trimethoprim resistance in Staphylococcus aureus.* Biochem J 1987;243:309-312.

CHAPTER 4

ANTIBIOTIC RESISTANCE IN HAEMOPHILUS INFLUENZAE

Mechanisms, clinical importance and consequences for therapy

This manuscript will be published as a review in Eur J Pediatr.

CHAPTER 4

ANTIBIOTIC RESISTANCE IN HAEMOPHILUS INFLUENZAE

Mechanisms, clinical importance and consequences for therapy

R. de Groot[1], G. Dzoljic-Danilovic[2], B. van Klingeren[3], W.H.F. Goessens[2], H.J. Neijens[1]

[1] *Department of Paediatrics, Division of Immunology and Infectious Diseases, Sophia Children's Hospital / University Hospital Rotterdam, The Netherlands*
[2] *Department of Microbiology, Erasmus University / University Hospital Rotterdam, The Netherlands*
[3] *National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands*

4.1 SUMMARY

Invasive and non-invasive infections caused by *Haemophilus influenzae* are frequently diagnosed in children below the age of 5 years. The treatment of choice for these infections was ampicillin. However, since the early 1970s the increasing prevalence of resistance to ampicillin and other antibiotics has necessitated major changes in antibiotic therapy. This article summarizes some of the important clinical features of diseases caused by *H. influenzae*. The epidemiology, the problems with in vitro susceptibility testing and the mechanisms of resistance to major antibiotics are reviewed. The consequences of antibiotic resistance for the treatment of diseases caused by *H. influenzae* are discussed.

4.2 INTRODUCTION

Haemophilus influenzae (H. influenzae) is an important micro-organism responsible for numerous infectious diseases such as meningitis, epiglottitis, cellulitis and upper respiratory tract infections with a peak incidence in children below the age of 5 years. *H. influen-*

zae can be classified into eight biotypes according to their metabolic properties [101]. The major virulence determinant of *H. influenzae* is the production of capsular polysaccharide [106]. Six different capsular types designated "a to f" have been described. Encapsulated strains (predominantly type b) are the causative agents of severe invasive infections, whereas unencapsulated *H. influenzae* are common pathogens in upper respiratory tract infections. Other components of the bacterial cell membrane such as pili, outer membrane proteins and lipopolysaccharides are also involved in the pathogenesis of invasive and non-invasive infections caused by *H. influenzae* [101].

Infections caused by *H. influenzae* type b (Hib) represent more than 90% of invasive diseases in young children [101]. The incidence of invasive Hib disease varies from 52/100,000 to 500/100,000 in children below 5 years of age [157]. Risk factors for Hib infection, the major diseases caused by Hib and the preferential choice of antibiotics in the treatment of Hib infections are summarized in Table 4.1.

Unencapsulated *H. influenzae* are frequently involved in the pathogenesis of otitis media, sinusitis, conjunctivitis, chronic bronchitis and bronchiectasis. Risk factors for infection caused by unencapsulated *H. influenzae*, the major infectious diseases and the preferential treatment of these infections are summarized in Table 4.2. Unencapsulated *H. influenzae* frequently cause pneumonia and concurrent bacteraemia in children in developing countries [9, 167]. However, encapsulated *H. influenzae* (serotype b) are more commonly isolated in sputum and blood cultures from patients with pneumonia [149, 167]. Recently a specific strain of unencapsulated *H. influenzae* (*Haemophilus aegyptius*) has been identified as the aetiological agent of a new recognized disease, Brazilian purpuric fever [53, 154]. The clinical presentation of Brazilian purpuric fever is identical to the features of the Waterhouse Fridericksen syndrome, but blood cultures grow *H. aegyptius* instead of *Neisseria meningitidis*. *H. aegyptius* was previously known as the cause of outbreaks of acute conjunctivitis in different parts of the world.

4.3 PREVENTION OF HIB INFECTION

Hib disease may be prevented by immunization with polysaccharide capsule (PRP) containing vaccines. The results of a large PRP vaccine trial in Finland indicated more than 90% protective efficacy in children older than 2 years of age [121]. Subsequent studies with PRP vaccines in different populations showed significantly less efficacy (45%-88%) [50]. PRP vaccine failures may have been due to differences in age distribution and genetic background between Finnish and American children of different racial groups [50, 157]. The recent development of polysaccharide conjugate vaccines, which confer protec-

Table 4.1 Major invasive diseases caused by encapsulated *H. influenzae*.

Risk factors	Age below 5 years Genetic predisposition B-cell deficiency Complement deficiency Sickle cell disease Absence of breast feeding		[40, 49, 60, 101, 158]
Diseases	Meningitis Bacteraemia Epiglottitis Septic Arthritis Pneumonia Osteomyelitis Pericarditis		[24, 103]
Preferential choice of antibiotics	Meningitis	Ampicillin[a] Cefotaxime Ceftriaxone Ceftazidime	[10, 54, 61, 75, 101, 116, 122, 135, 139]
	Pneumonia	Ampicillin[a] Trimethoprim-sulphamethoxazole Amoxicillin-clavulanate Cefuroxime	[22, 101, 112, 142, 165]
	Other infections	Ampicillin[a] Cefuroxime	[101]

[a] Ampicillin IV is first choice agent in the treatment of ampicillin-susceptible *H. influenzae*.

tion to a much younger age, are promising [37]. These vaccines contain a combination of PRP and carrier proteins such as diphtheria toxoid (PRP-D vaccine), outer membrane protein of meningococci (PRP-OMP vaccine), tetanus toxoid (PRP-T vaccine) or a variant of diphtheria toxin (HbOC vaccine) [4, 45, 84, 143]. The combination of carrier proteins and PRP induces a T-cell dependent immune response, which leads to the production of protective IgG levels (> 1.0 μg/ml) of PRP antibodies in infants of 6 months and older [67, 145].

Table 4.2 Diseases caused by unencapsulated *H. influenzae*.

Risk factors	Diseases	Preferential choice of antibiotics	References
Age below 3 years Familial disposition	Otitis media Sinusitis Conjunctivitis	Amoxicillin[a] Amoxicillin-clavulanate Trimethoprim-sulphamethoxazole Cefuroxime	[5, 13, 36, 38, 85, 86, 87, 101, 109, 115, 144, 147, 155]
Elderly people Chronic bronchitis Impaired immunity HIV infection in young adults Malnutrition Social deprivation	Pneumonia	Amoxicillin[a] Amoxicillin-clavulanate Trimethoprim-sulphamethoxazole Cefuroxime	[9, 101, 109, 141, 149, 163, 168]
Prematurity Chorioamnionitis Prolonged rupture of membranes	Neonatal septicaemia	Amoxicillin[a] Cefotaxime	[41, 109, 164]
Cystic fibrosis Bronchiectasis Chronic obstructive pulmonary disease	Chronic respiratory tract infection	Amoxicillin[a] Amoxicillin-clavulanate Trimethoprim-sulphamethoxazole	[109]
Recent conjunctivitis	Brazilian purpuric fever	Amoxicillin[a] Chloramphenicol	[53, 154]

[a] Amoxicillin PO or ampicillin IV are first choice agents in the treatment of ampicillin-susceptible *H. influenzae*.

4.4 ANTIBIOTIC THERAPY OF *H. INFLUENZAE* INFECTION

In the early 1970s the treatment of choice for infections caused by *H. influenzae* was ampicillin (Ap). The increasing prevalence of antibiotic resistance to Ap and other antibiotics in both unencapsulated and encapsulated *H. influenzae* has necessitated changes in the initial antibiotic treatment of invasive and non-invasive infections (see Tables 4.1 and 4.2).

4.5 IN VITRO SUSCEPTIBILITY TESTING

The increasing prevalence of antibiotic resistance in *H. influenzae* necessitates routine susceptibility testing for several clinically important antibiotics [32]. However, in vitro susceptibility testing especially in *H. influenzae* is complicated because minimum inhibitory concentration (MIC) values are influenced by the composition, pH and osmolarity of growth media, the size of the inoculum used, the percentage of CO_2 during incubation and inhibition of certain antibiotics by constituents in media. In addition, in vitro results do not always correlate with in vivo efficacy. The lack of standardization of in vitro tests is reflected by the presence of substantial differences between the MIC breakpoints in recent studies (Table 4.3). Similar difficulties exist in the interpretation of growth end points in disk diffusion susceptibility testing [31, 104]. The prevalence of resistance to the antibiotics mentioned in Table 4.3 is discussed in the following sections. Routine MIC testing may also be useful for second generation cephalosporins such as cefuroxime which are commonly used in the treatment of *H. influenzae* infections. In vitro susceptibility testing of *H. influenzae* for third generation cephalosporins (including cefotaxime, ceftazidime and ceftriaxone), the carbapenem imipenem, the monobactam aztreonam, the

Table 4.3 Susceptibility testing in *Haemophilus influenzae*.

	MIC breakpoints (μg/ml) in different studies							
	Susceptible				Resistant			
Antibiotic	a	b	c	d	a	b	c	d
Ampicillin	2	< 1	2	2	4	> 4	4	4
Chloramphenicol	8	< 2	2	4	32	> 8	4	8
Erythromycin	0.5	-	4	0.5	8	-	8	1
Trimethoprim	2	< 0.5	-	0.5	4	> 4	-	4
Cefaclor	8	< 8	8	8	32	> 32	16	32
Rifampin	2	< 1	-	2	8	> 4	-	8
Tetracycline	4	< 2	2	4	16	> 8	4	16

References: a [111]; b [33]; c [81]; d [64]

combination of amoxicillin with clavulanic acid and the oral cephems (e.g. cefixime, ceftibuten and cefpodoxime) indicates, that these new β-lactams have an excellent in vitro activity against *H. influenzae* [8, 18, 29, 32, 73, 103, 126, 151]. However, the recent emergence of Ap-resistant non-β-lactamase producing *H. influenzae* may also result in an increasing prevalence of resistance to these β-lactams (see section on cephalosporins).

4.6 RESISTANCE TO AMPICILLIN (AP)

Ap-resistant *H. influenzae* strains were first isolated in 1974 from blood, CSF or pus from patients with sepsis, meningitis, otitis media and from nasopharyngeal carriers of *H. influenzae* [21, 69, 140, 159, 161]. Since then, Ap resistance in both classified and non-classified strains of *H. influenzae* has increased and has become a major problem in many European countries and the United States of America. Doern et al. reported in a national collaborative study on antibiotic resistance of *H. influenzae* a 31.7% prevalence of Ap resistance in classified strains and a 15.6% prevalence in non-classified strains [33]. A recent collaborative European study shows large differences in prevalence between individual countries. The highest prevalence of Ap resistance was found in Spain (30.6%) and the lowest in the Federal Republic of Germany (1.6%) with a mean value of 10% for all participating countries [81]. The percentage of Ap resistance in classified and non-classified *H. influenzae* was 14% (range 0%-64%) and 9% (range 2%-26%) respectively [81].

Ap resistance in *H. influenzae* is in more than 90% of isolates caused by the production of a (TEM)type β-lactamase [27]. ß-lactamase can be detected by a rapid screening test [160]. The enzyme hydrolyzes penicillins and most of the first generation cephalosporins [94]. The gene encoding TEM β-lactamase is located on a 3 x 10^6 dalton sequence of plasmid DNA, called transposon A (TnA) [57]. The ability of TnA to translocate from one replicon to another has been extensively characterized [55, 56]. TnA is found on many different plasmids in Gram-negative bacteria [57]. One might speculate, that TnA derived from another Gram-negative micro-organism has translocated to cryptic plasmids in *H. influenzae*, thus leading to Ap resistance. TEM β-lactamase inducing Ap resistance in *H. influenzae* is in most strains encoded on large ± 30 x 10^6 dalton (30 Mda) plasmids [34, 35, 74, 138]. However, some *H. influenzae* strains only contain small ± 3 Mda plasmids, which may also encode Ap resistance [34, 48, 153]. In addition, Mendelman et al. have reported, that β-lactamase-mediated Ap resistance in *H. influenzae* strains isolated from native Alaskans is primarily due to a common 40 Mda conjugative plasmid [99]. The gene encoding resistance to Ap in these strains probably also contains TnA.

Although plasmid-mediated β-lactamase production is the most common mechanism of Ap resistance in *H. influenzae*, chromosomal-mediated resistance to Ap has also been described. This type of resistance is presumably caused by transfer of β-lactamase genes from plasmid to chromosome through a process of recombination [146, 168]. In one study approximately 8% of Ap-resistant β-lactamase producing isolates of *H. influenzae* was found to produce a (ROB-1) β-lactamase [27]. Rubin et al. were the first to report production of ROB-1 by an isolate from a patient with meningitis [136]. The gene encoding this enzyme and the resulting protein are not related to the TEM-1 gene and enzyme [27, 76, 95]. ROB-1 β-lactamase production in *H. influenzae* is probably transferred by small (4.4 Mda) plasmids [27]. Medeiros et al. suggested, that ROB-1 may have spread from an animal reservoir to humans [95]. The report of Livrelli et al. on the production of ROB-1 β-lactamase in several bovine strains of *Pasteurella sp.* further supported this hypothesis [80]. Recently the nucleotide sequence of the ROB-1 β-lactamase gene from *H. influenzae* has been determined [65]. The deduced amino acid sequence shares the highest similarity with the class A β-lactamases of Gram-positive bacteria [65]. This suggests, that the ROB-1 gene may have been acquired from Gram-positive micro-organisms.

In the last decade non-β-lactamase producing *H. influenzae* strains with decreased susceptibility to Ap have been characterized [88, 97, 102, 117]. The majority of these *H. influenzae* is non-classified [102]. Mendelman et al. have shown a large genetic and phenotypic diversity between these isolates from different countries [102]. The detection of these strains has probably been complicated by the fact, that their MICs (1-4 μg/ml) are only slightly above those of susceptible strains (0.25-0.5 μg/ml) [124, 128] and by the inadequacy of standard susceptibility testing [100, 104]. The mechanism of this moderate level of resistance to β-lactam antibiotics is a chromosomally mediated alteration in penicillin-binding proteins 3A and 3B [97, 119]. The genes encoding this type of resistance have been cloned [82]. It is most likely, that mutations in one or more of these genes lead to a marked decrease in affinity of penicillin-binding proteins 3A en 3B for different β-lactam antibiotics. These mutations probably involve different genetic loci since a substantial variability exists between the MICs of different isolates for Ap and other β-lactam antibiotics.

4.7 RESISTANCE TO CEPHALOSPORINS

Cephalosporins are increasingly used in the treatment of invasive and non-invasive infections caused by *H. influenzae*. However, the limited susceptibility of *H. influenzae* for many first-generation cephalosporins and the poor CSF penetration of these drugs restrict their application to non-invasive infections. The most active of the older oral cephalosporins is cefaclor [33, 123]. Using the MIC interpretative standards of the National Committee

America and ± 98% from Europe, were susceptible (MIC ≤ 8 μg/ml) [33, 81]. However, most of these isolates have relatively high MICs varying between 2 and 8 μg/ml [33, 81, 123]. The in vitro susceptibility of *H. influenzae* for second generation cephalosporins including cefamandole and cefuroxime is adequate [123]. Nevertheless, clinical failures with children developing *H. influenzae* meningitis, while treated with cefamandole for other *H. influenzae* infections have occurred [150]. This may be explained by the insufficient CSF levels of cefamandole in the presence of meningeal inflammation. In addition, recent studies have indicated that patients with *H. influenzae* meningitis treated with cefuroxime intravenously, show a delayed sterilization of CSF and a higher incidence of neurological sequelae compared to children treated with third-generation cephalosporins [75, 139]. Third-generation cephalosporins including cefotaxime, ceftazidime, ceftriaxone and moxalactam are extraordinarily active against *H. influenzae* with MICs ranging from 0.004 to 0.25 μg/ml [103, 123].

Recently a new group of oral third generation cephalosporins (cephems) including cefixime, ceftibuten and cefpodoxime has been developed. These agents are highly active in vitro against *H. influenzae* whereas their activity is unaffected by β-lactamase [73, 103, 125]. The clinical efficacy of these compounds in the treatment of *H. influenzae* infections remains to be shown. Ap-susceptible and Ap-resistant β-lactamase producing *H. influenzae* have approximately equal MICs for the cephalosporins [103, 123]. Decreased susceptibility to cephalosporins is therefore mainly seen in non-β-lactamase producing (NBLP) Ap-resistant *H. influenzae*. Currently most of the cephalosporin-resistant *H. influenzae* are isolated from the respiratory tract of patients with chronic pulmonary disease e.g. cystic fibrosis, chronic obstructive pulmonary disease or chronic bronchitis [128]. The prevalence of NBLP Ap-resistant *H. influenzae* isolates is unknown, but probably less than 5% [128]. However, the detection of these strains has been complicated by problems with in vitro susceptibility testing. Doern et al. [31] and Mendelman et al. [100, 104] have recently reported improved laboratory methods to identify these strains. Powell et al. have indicated, that the resulting accurate detection of Ap-resistant NBLP *H. influenzae* gives an indication of the prevalence of cephalosporin resistance in *H. influenzae* [128]. The mechanism of resistance in these isolates is probably identical to the mechanism described in Ap-resistant NBLP *H. influenzae*, e.g. an alteration in the affinity of penicillin-binding proteins for different cephalosporins. The degree of heterogeneity of MICs and the genetic and phenotypic diversity of these strains suggest that different genetic mutations may be involved. The increased use of cephalosporins in the therapy of diseases caused by *H. influenzae* may easily lead to an increased selection of these isolates.

4.8 RESISTANCE TO CHLORAMPHENICOL

Chloramphenicol (Cm) resistance in *H. influenzae* was first described in 1976 [83]. Although the prevalence of Cm resistance remains below 1% in the United States of America and several European countries, Cm resistance has become a major problem in Spain (24.9%) and Belgium (10.9%) [81]. Cm-resistant strains are often additionally resistant to Ap, tetracycline (Tc), and the combination of trimethoprim/sulphamethoxazole (Tmp/Smz) [20, 81, 124, 152, 166]. Cm resistance occurs in more than 90% via the production of chloramphenicol acetyltransferase (CAT) [14, 131]. CAT can be detected by a rapid screening test [6]. CAT catalyzes the diacetylation of Cm with acetyl-CoA thus preventing the inhibition of ribosomal protein synthesis. Characterization of CAT from different *H. influenzae* strains has shown that despite differences these enzymes belong to the same unique group and are related to the type II enzymes in *Escherichia coli* [132]. Cm resistance caused by CAT is encoded on large (30-50 Mda) plasmids, which may be easily transferred by conjugation and commonly integrate into the chromosome [20, 70, 98, 127]. Cm-resistant *H. influenzae* strains, which do not produce CAT have been recognized [14]. However, their prevalence is very low (± 7%) [14, 123]. The mechanism of Cm resistance in these strains is a decreased permeability of the bacterial cell wall due to the loss of an outer membrane protein [14].

4.9 RESISTANCE TO TRIMETHOPRIM

Trimethoprim (Tmp) is a commonly used antibiotic in the treatment of upper respiratory tract infections such as otitis media and sinusitis, often caused by unencapsulated *H. influenzae* [38]. Routine susceptibility testing for Tmp in *H. influenzae* has been complicated by antagonism of Tmp by thymidine present in many commercial media [12, 30, 32, 63]. This may lead to the erroneous detection of resistant strains. The prevalence of Tmp resistance in the United Kingdom has increased from 0.2% in 1977 to 4.2% in 1986 [124]. Yogev et al. have shown, that some type b *H. influenzae* strains colonizing the nasopharynx, persist after therapy with Tmp/Smz [171]. A proportion of these strains are susceptible to Tmp/Smz in MIC testing (MIC $\leq$ 0.03/0.6 µg/ml) but have high minimal bactericidal concentrations ($\geq$ 4/76 µg/ml). These *H. influenzae* are called "tolerant". Tolerant isolates were found to produce significantly more capsular polysaccharide than strains with normal MIC and minimal bactericidal concentration values (susceptible isolates). Animal studies using both types of isolates indicated that tolerant strains were pathogenic, whereas susceptible *H. influenzae* with less polysaccharide capsule could not cause systemic disease. The mechanism of tolerance to the lethal action of Tmp/Smz still waits to be elucidated.

There is a lack of information on the prevalence of Tmp resistance in *H. influenzae* in

many countries, since most studies only report MICs for the combination of Tmp and Smz. The national collaborative study in the United States of America registered 0.9% resistant *H. influenzae* (MIC for Tmp/Smz ≥ 1.0/19 μg/ml). In contrast, resistance to Tmp is a major problem in Spain [15, 17, 19]. Tmp resistance in these Spanish isolates is frequently associated with plasmid-encoded resistance to Ap, Cm and kanamycin (Km) [20]. However, Tmp resistance in these encapsulated strains, but also in unencapsulated *H. influenzae* from different geographic areas is chromosomally encoded [51]. The gene encoding Tmp resistance in *H. influenzae* has been cloned and expressed in *E. coli* [51]. The mechanism of Tmp resistance in *H. influenzae* is a combination of overproduction of the target enzyme dihydrofolate reductase and structural and functional differences between dihydrofolate reductase from susceptible and resistant strains [52].

4.10 RESISTANCE TO ERYTHROMYCIN

The macrolide erythromycin (Em) is commonly used in the treatment of upper respiratory tract infections. Susceptibility testing of macrolide antibiotics against *H. influenzae* shows substantial differences depending on the method employed [39]. Using criteria of the National Committee for Clinical Laboratory Standards Doern et al. reported, that only 50% of 2811 clinical *H. influenzae* isolates was susceptible (MIC breakpoint 8 μg/ml) to Em [33]. Most strains in this study had MICs varying between 2 and 8 μg/ml [33]. The results of a European cooperative study in which a different MIC breakpoint (4 μg/ml), different media and a different inoculum were used, show that Em resistance ranged from 27% of isolates in The Netherlands to 1.1% in Austria [81]. The data indicate that Em resistance in *H. influenzae* is more prevalent in unencapsulated than in encapsulated strains. In addition, the large number of isolates with relatively high MICs (1-8 μg/ml) suggests a limited potential for the use of Em in the therapy of *H. influenzae* infections. Several studies have indicated, that the combination of Em with different sulfonamides is as effective as amoxicillin or cefaclor in curing patients with acute otitis media caused by Ap-susceptible or Ap-resistant *H. influenzae* [11, 58, 133, 134]. However, amoxicillin and Tmp/Smz have significantly better penetration into the middle ear [71]. In addition, the ratio of mean peak concentration in middle ear fluid to the MIC for *H. influenzae* is also much higher when these antibiotics are compared with Em. The availability of additional β-lactams such as amoxicillin-clavulanate (augmentin) and cefuroxime axetil, which are both very effective against β-lactamase and non-β-lactamase producing *H. influenzae*, limits the use of Em in our opinion to those patients with otitis media whereby β-lactams or Tmp/Smz are contraindicated (see also discussion). Ringertz and Kronvall reported that Em resistance is associated with an increased drug consumption [130]. The mechanism of action of Em is the inhibition of bacterial protein synthesis by blocking the function of the 50S ribosomal subunit. The mechanism of Em resistance in *H. influenzae* is not known

and it is still uncertain whether the resistance markers are plasmid- or chromosomally encoded.

4.11 RESISTANCE TO TETRACYCLINE

Tetracycline (Tc) is a bacteriostatic antibiotic, which inhibits protein synthesis by binding to the 30S prokaryotic ribosomal subunit [28]. Tc resistance in *H. influenzae* was first recognized in 1975 [25]. The prevalence of Tc resistance remains below 5% in the United States of America and most European countries [33, 81]. Tc resistance is frequently present in combination with plasmid-encoded resistance to Ap and Cm [18]. Tc resistance is considerable in Spain (25.4%) and Belgium (17.8%) where multiple-resistant *H. influenzae* are common [81]. Tc resistance is encoded on 30-40 Mda conjugative plasmids [35, 62, 66]. These plasmids contain inverted repeats and DNA sequences largely homologous with the Tc translocation segment transposon TnTc or Tn10 [62]. Tn10 is found on plasmids in members of the family *Enterobacteriaceae*. Four different classes (A, B, C and D) of plasmid-mediated Tc resistance have been described [105]. McMurry et al. have shown that the mechanism of Tc resistance in *E. coli* in each of these classes is an increase in the energy-dependent efflux system of Tc out of the bacterial cell [93]. Tc resistance in *H. influenzae* is predominantly associated with the class B determinant of Tn10 [89]. The level of Tc resistance in *H. influenzae* can be increased by the addition of Tc to culture media. The constitutive expression of Tc resistance has been studied in *H. parainfluenzae* and is probably caused by the inactivation of the repressor molecule for the Tc transmembrane transport protein [78, 89].

4.12 RESISTANCE TO AMINOGLYCOSIDES

The availability of many antibiotics with high in vitro activity against *H. influenzae* and relatively minor side-effects has limited the study of resistance of *H. influenzae* to aminoglycosides. The prevalence of resistance to aminoglycosides in *H. influenzae* is therefore unknown. Dang Van et al. were the first to report a Km-resistant *H. influenzae* [26]. Resistance in this strain could be transferred by conjugation, suggesting that the mechanism of resistance was plasmid-mediated. We recently showed that resistance to streptomycin (Sm) and Km in multiple-resistant *H. influenzae* from different geographic areas in Spain is common and plasmid-encoded [20]. The mechanism of Km resistance is modification of the drug by aminoglycoside-phosphotransferase(3')I.

Levy et al. studied aminoglycoside resistance in clinical isolates from the United States of America [79]. They suggested that frequent use of aminoglycosides in patients with cystic fibrosis or chronic respiratory tract infections could select resistant strains. They reported

that 6 out of 38 strains isolated from lower respiratory tract infections were resistant to a broad range of aminoglycosides including tobramycin, gentamicin, amikacin, sisomicin and kanamycin, whereas none of 63 strains isolated from other body sites was resistant. They investigated the mechanism of resistance and reported in contrast to Campos et al. [20], that resistance was chromosomally encoded. Resistance was not caused by aminoglycoside modifying enzymes but by ribosomal alteration of protein synthesis.

4.13 RESISTANCE TO QUINOLONES

Quinolones are increasingly used in the treatment of a variety of infections in adults and have been suggested as useful agents in the treatment of infections caused by *H. influenzae* [113]. Studies in the murine model of bacteraemic Hib pneumonia have shown that quinolones such as enoxacin and ofloxacin are effective antibiotics in the intrapulmonary killing and treatment of Ap-susceptible and Ap-resistant strains [68]. The use of quinolones in paediatric patients has been limited due to the documented irreversible damage to cartilage in strained joints of young animals [1]. Bacterial resistance to *H. influenzae* is either due to alteration of the target enzyme DNA gyrase (an enzyme necessary for DNA replication) or due to decreased drug permeation of the bacterial cell [169]. Quinolone-resistant *H. influenzae* have not yet been reported.

4.14 RESISTANCE TO RIFAMPIN

Chemoprophylaxis with rifampin (Rif) (20 mg/kg once daily for 4 days) of household contacts of children with invasive Hib infection is recommended by most experts [129]. Chemoprophylaxis should be used for all household contacts when there is a child less than 4 years of age other than the index patient. However, only one prospective study has evaluated the effect of Rif prophylaxis and the results of this study have been criticized because of methodological errors [7, 108]. Two recent studies indicate, that the risk of subsequent disease in contacts of patients in day-care facilities is significantly lower than previously reported and may be similar to the base rate of primary disease in day-care facilities [110, 118]. Therefore prior recommendations to administer Rif to day-care contacts of children with invasive disease have become controversial [129]. Rif eradicates nasopharyngeal carriage of *H. influenzae* day-care contacts and household contacts [23, 44]. However, new acquisition of Hib continues to occur at a low rate among those, who have received Rif prophylaxis [44]. Nicolle et al. have reported, that prophylactic administration of Rif to children and adults with nasopharyngeal isolates of *H. influenzae* leads to the rapid emergence of resistant strains [114]. These isolates had two levels

of MIC: intermediate (MIC 1-10 μg/ml) and high (MIC > 128 μg/ml). The in vitro mutation frequency of *H. influenzae* to Rif resistance is approximately 10^{-8} [96]. The prevalence of Rif resistance in clinical isolates is below 1% [33], although one has to be concerned about the future implications of the high in vitro and in vivo mutation frequency to Rif resistance. An alternative to the use of Rif could be the combination of Rif and Tmp in the treatment of carriers of *H. influenzae*. This combination at a 1:1 ratio is synergistic and bactericidal [92]. Rif has seldomly been used for the treatment of invasive or non-invasive infections by *H. influenzae*. A recent study reported in vitro synergy against Hib of several combinations of Rif with cephalosporins [46]. Although these in vitro data suggest a potential role for Rif in the treatment of some cases of Hib meningitis, we feel, that future clinical studies with the combination of Rif and cephalosporins are not warranted in view of the abundancy of alternative antibiotics (see sections on cephalosporins and quinolones) and the high in vitro mutation frequency.

The mechanism of Rif resistance in *H. influenzae* is unknown. Resistant isolates show several degrees of sensitivity to Rif, indicating that different mechanisms may be involved. Most likely a change in the structure of RNA polymerase, which leads to a decreased binding of the polymerase to Rif, is responsible for resistance [72]. Alternatively a change in membrane permeability of Rif might be involved [72].

4.15 MULTIPLE-RESISTANT H. INFLUENZAE

The emergence of Hib resistant to Ap and Cm in patients with meningitis was first reported in Thailand and California in 1980 [148, 162]. Ap- and Cm-resistant Hib meningitis in Europe was initially diagnosed in 1983 [42]. Campos et al. subsequently showed, that multiple-resistant Hib had become a major problem in Spain [15, 16]. Approximately 50% of isolates from patients with invasive disease or from *H. influenzae* carriers in the Barcelona area were resistant to multiple antibiotics. The most frequent pattern of resistance was resistance to Ap, Cm, Tc, Tmp/Smz (94.8%). The prevalence of multiple-resistant *H. influenzae* in Spain is currently approximately 25.4%, although considerable regional differences are detected [81]. The same study also reported a high prevalence of multiple-resistant *H. influenzae* in Belgium (9.4%) [81]. Multiple-resistant *H. influenzae* are less common in other European countries and in the United States of America [33, 81]. The prevalence of resistance to multiple antibiotics is ± twice as high in encapsulated compared to unencapsulated *H. influenzae* strains [81]. Multiple-resistant *H. influenzae* harbour plasmids with molecular sizes of 38-52 Mda [19, 20, 99]. These plasmids may be transferred to recipient *H. influenzae* by conjugation or transformation [20]. Conjugative plasmids contain genes encoding resistance to Ap, Cm, Tc, Km, Sm and Smz [20]. In contrast, Tmp resistance is not plasmid-mediated, but chromosomally encoded [51]. The mechanisms of resistance in multiple-resistant *H. influenzae* are similar to those described

in the previous sections.
Campos et al. questioned whether the dissemination of multiple-resistant Hib in Spain was caused by a single R-plasmid [19]. Their study using isoenzyme analysis, plasmid restriction endonuclease analysis and determination of outer membrane profiles indicated that more than one endemic strain of Hib was responsible for the appearance of multiple-resistant Hib disease in day-care centres. Van Alphen et al. subsequently compared antibiotic-resistant and -susceptible isolates of Hib from The Netherlands [3]. Of the resistant strains 84%, as compared to 80% of susceptible strains had a similar major outer membrane profile (subtype 1), biotype (nr 1) and lipopolysaccharide (nr 1) [2, 3]. These results indicate that resistance to antibiotics is not spread through the dissemination of one clone, but develops at random. It remains to be determined if more appropriate use of antibiotics may decrease the random selection of highly resistant *H. influenzae*. However, retrospective analysis of prior use of Ap and Tc in children and adults with Ap- and multiple-resistant *H. influenzae* indicates, that the emergence of resistance is associated with prior use of antibiotics [156, 166].

4.16 CLINICAL IMPLICATIONS

H. influenzae is still a major cause of invasive and non-invasive infections in young children. The widespread use of conjugate vaccines may in the near future lead to the eradication of invasive disease caused by *H. influenzae* type b. However, antibiotic resistance will remain a major problem in unencapsulated strains since the perspective for vaccination against these isolates is still remote. The emergence of resistance to common antibiotics e.g. Ap and Cm in many European countries and the United States of America has resulted in clinical failures when these antibiotics were used. The increase of Ap-resistant β-lactamase producing unencapsulated strains causing otitis media has resulted in an increased application of alternative antibiotics including amoxicillin-clavulanate (augmentin), erythromycin-sulfisoxazole, trimethoprim-sulphamethoxazole, cefaclor and cefuroxime axetil [13]. Marchant et al. critically analysed 25 trials on antibacterial therapy for otitis media [85]. In 21 of the 25 studies no significant differences were found between two or more antibiotics. The authors postulated, that methodological problems in study designs might make trials insensitive. These include the absence of an appropriate microbiological diagnosis, the application of insensitive outcome criteria such as persistent middle ear effusion, the lack of documentation of eradication of micro-organisms from the middle ear and an insufficient sample size of the study. The evaluation of efficacy of antibiotics in patients with acute otitis media is further complicated by the high percentage of spontaneous recovery due to the contribution of host defences. Four studies have evaluated the efficacy of cefaclor compared with Tmp/Smz, amoxicillin, augmentin and cefuroxime in the treatment of acute otitis media [5, 86, 87, 115]. Two of these studies

showed significantly better eradication of micro-organisms including *H. influenzae* despite a failure to detect differences in clinical efficacy [86, 87] The two other studies reported better clinical efficacy of augmentin and cefuroxime compared to cefaclor [5, 115]. These results are not surprising in view of the relatively high MIC of *H. influenzae* and other pathogens for cefaclor and the poor penetration of cefaclor in middle ear fluid [71]. Also a marked inoculum effect has been observed in in vitro studies of the antibacterial effect of cefaclor [77]. With inocula in excess of 10^6 colony-forming units per ml Hib is neither inhibited nor killed by cefaclor at concentrations of 400 μg/ml [77]. This may be important since the inoculum size in an infected middle ear may be as high as 10^8 micro-organisms per gram of exudate [59]. The use of cefaclor as a first choice or alternative agent in the treatment of acute otitis media is not justified in our opinion in view of the superior bacteriological and clinical efficacy of several other antibiotics e.g. augmentin, cefuroxime and Tmp/Smz. The use of Em-sulfisoxazole should be restricted to patients with otitis media, who can not be treated with β-lactams or Tmp/Smz (see section on Em). The efficacy of augmentin, cefuroxime and Tmp/Smz in the treatment of Ap-resistant *H. influenzae* otitis media and sinusitis needs to be proven in prospective comparative randomized studies, although these antibiotics are efficacious in open trials [36, 144, 155].

The increasing prevalence of Ap and Cm resistance in encapsulated *H. influenzae* and the documented failure of Ap therapy in patients with invasive infections caused by Ap-resistant *H. influenzae* are partly responsible for the widespread use of broad spectrum β-lactam antibiotics especially in *H. influenzae* meningitis. Some patients with Ap-resistant strains causing epiglottitis, cellulitis, pneumonia and arthritis appear to react to Ap in vivo [43, 107]. The presence of an in vivo response despite poor in vitro susceptibility may be explained by factors such as the inoculum effect, the contribution of host defences to cure and the possibility, that a high dosage of Ap may quantitatively overcome β-lactamase levels. However, we advise treatment of infections with Ap-resistant Hib with β-lactamase stable antibiotics in view of the expected, but not documented superiority in clinical and bacteriological efficacy. Both augmentin and cefuroxime appear to be effective antibiotics in the treatment of lower respiratory tract infections by Ap-susceptible and -resistant *H. influenzae* [22, 112, 142, 165]. The efficacy of ceftriaxone and cefotaxime compared to Ap and Cm in the treatment of meningitis has been established in prospective randomized studies [10, 61, 116, 122, 139]. Recent studies indicate, that ceftriaxone is superior to cefuroxime (a second generation cephalosporin) because of a more rapid sterilization of CSF and a decreased percentage of patients with hearing impairment [75, 139]. Other β-lactam antibiotics, which show excellent in vitro and in vivo activity in animal models of experimental *H. influenzae* meningitis include aztreonam, ceftazidime and imipenem [90, 91, 137]. The efficacy of ceftazidime in the treatment of meningitis caused by *H. influenzae* has been proven in several studies [54, 135]. However, additional study is necessary to define the role of ceftazidime in meningitis caused by *S. pneumoniae* or *N. meningitidis*. The use of imipenem-cilastatin in patients with bacterial meningitis has

been associated with a high incidence of seizures [170]. The efficacy of aztreonam needs to be proven in comparative studies of children with meningitis.

Routine susceptibility testing of *H. influenzae* isolates will remain necessary in order to detect resistance to clinically important antibiotics. Standardization of in vitro testing may lead to a more reliable comparison of the prevalence of antibiotic resistance in different countries and a more accurate detection of the different types of resistance, especially resistance to Ap. Ap-resistant *H. influenzae*, but also intermediate susceptible *H. influenzae* (MIC between 2 and 8 μg/ml) need to be tested for the presence of β-lactamase. The detection of Ap-resistant NBLP *H. influenzae* may serve as an indicator for the presence of decreased susceptibility to β-lactam antibiotics. The current recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) for susceptibility testing in *H. influenzae* have recently been shown to misclassify Ap-resistant NBLP strains as intermediate or susceptible [104]. In addition, the NCCLS medium did not support the growth of a substantial number of AP-resistant NBLP *H. influenzae* [104]. This may partly be due to the auxotrophic requirements of these strains [102]. The fastidious growth of especially non-typeable *H. influenzae* isolates from respiratory sites necessitates the use of rich media such as Mueller-Hinton chocolate agar for MIC testing. Susceptibility testing needs to be performed with freshly prepared standard inocula (e.g. 10^5 CFU) by agar dilution tests. Disk susceptibility testing for Ap should be performed with 2 μg Kirby-Bauer Ap disks [104]. The detection of Ap-resistant β-lactamase producing *H. influenzae* indicates the presence of R-plasmids, which may also carry other antibiotic resistance genes (Cm, Tc, aminoglycosides). The mechanisms of antibiotic resistance described in the previous sections have been summarized in Table 4.4.

Plasmid-mediated resistance to Ap and the multiple-resistant *H. influenzae* strains containing plasmids encoding resistance to Ap, Cm and Tc will probably become increasingly prevalent in both children with invasive and non-invasive disease unless proper measures are taken. Recent outbreaks of multiple-resistant unencapsulated *H. influenzae* infections in adult patients with chronic obstructive pulmonary disease, Ap-resistant encapsulated *H. influenzae* infections in geriatric patients and Ap-resistant unencapsulated *H. influenzae* infections in an acute medical chest ward have focused attention on the additional potential of these strains to cause disease in adults [47, 120, 152]. We presume, that selection and subsequent spread of resistant isolates may preferably happen in unencapsulated *H. influenzae* from patients with chronic respiratory disease. These patients are recurrently and often treated with antibiotics for prolonged periods thus increasing the risk for selection of resistant isolates. Wallace et al. have shown in adults with chronic lung disease, that patients with unencapsulated *H. influenzae* resistant to Ap, Cm or Tc were more likely to have received prior antibiotics, than control subjects [166].

Table 4.4 Mechanisms of antibiotic resistance in *Haemophilus influenzae*.

Antibiotic	Mechanisms of resistance	R-plasmid- or Chromosomal-mediated
Ampicillin	- TEM-1 β-lactamase - ROB-1 β-lactamase - Mutation in penicillin-binding proteins	- 3, 30 or 40 Mda plasmid - 4.4 Mda plasmid - Chromosomal
Cephalosporins	- Mutation in penicillin-binding proteins	- Chromosomal
Chloramphenicol	- Chloramphenicol acetyltransferase - Decreased permeability	- 30-50 Mda plasmid - Chromosomal
Trimethoprim	- Overproduction of dihydrofolate reductase (DHFR) - Structural and functional alteration of DHFR	- Chromosomal
Erythromycin	- Probably inhibition of ribosomal protein synthesis	- Unknown
Tetracycline	- Probably inactivation of repressor molecule for Tc transmembrane transport protein	- 30-40 Mda plasmid
Aminoglycosides		
Kanamycin	- Aminoglycoside-phosphotransferase(3')I	- Plasmid
Tobramycin Gentamicin Amikacin Sisomicin Kanamycin	- Ribosomal alteration of protein synthesis	- Chromosomal
Rifampin	- Probably decreased binding of rifampin to RNA polymerase or decreased membrane permeability of rifampin	- Chromosomal

Chromosomally mediated resistance to Ap is less prevalent and increases more slowly than plasmid-mediated resistance. As shown before, this type of resistance is caused by mutations in the penicillin-binding proteins of *H. influenzae*. As a result, MICs for many β-lactam antibiotics are significantly increased, which may even result in resistance to third-generation cephalosporins.

We suggest, that a future spread of antibiotic resistance in *H. influenzae* may be halted or delayed by a more restricted use of antibiotics and continuous epidemiological monitoring.

4.17 REFERENCES

1 **Adam D.** *Use of quinolones in pediatric patients.* Rev Infect Dis 1989;11:S1113-S1116.

2 **Alphen L. van, Riemens T., Poolman J., Hopman C., Zanen H.C.** *Homogeneity of cell envelope protein subtypes, lipopolysaccharide serotypes, and biotypes among Haemophilus influenzae type b from patients with meningitis in The Netherlands.* J Infect Dis 1983;148:75-81.

3 **Alphen L. van, Bol P., Arends A., Riemens T., Geelen L.** *Comparison of antibiotic resistant and sensitive strains of Haemophilus influenzae type b in The Netherlands by outer-membrane protein subtyping.* Eur J Clin Microbiol Infect Dis 1988;7:309-311.

4 **Anderson P.W., Pichichero M.E., Insel R.A., Betts R., Eby R., Smith D.H.** *Vaccines consisting of periodate-cleaved oligosaccharides from the capsule of Haemophilus influenzae type b coupled to a protein carrier: structural and temporal requirements for priming in the human infant.* J Immunol 1986;137:1181-1186.

5 **Aronovitz G.H.** *Treatment of otitis media with cefuroxime axetil.* Southern Med J 1988;81:978-980.

6 **Azemun P., Stull T.L., Roberts M.C., Smith A.L.** *Rapid detection of chloramphenicol resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1981;20:168-170.

7 **Band J.D., Fraser D.W., Ajello G.W.** *Prevention of Haemophilus influenzae type b disease.* JAMA 1984;251:2381-2386.

8 **Bannatyne R.M., Toma S., Cheung R., Hodge D.** *Antibiotic susceptibility of blood and cerebrospinal fluid isolates of Haemophilus influenzae.* J Antimicrob Chemother 1985;15:187-191.

9 **Barker J., Gratten M., Riley I., Lehmann D., Montgomery J., Kajoi M., Gratten H., Smith D., Marshall T.F.C. Alpers M.P., de.** *Pneumonia in children in the Eastern Highlands of Papua New Guinea: a bacteriologic study of patients selected by standard clinical criteria.* J Infect Dis 1989;159:348-352.

10 **Barson W.J., Miller M.A., Brady M.T., Powell D.A.** *Prospective comparative trial of ceftriaxone vs. conventional therapy for treatment of bacterial meningitis in children.* Pediatr Infect Dis J 1985;4:362-368.

11 **Bergeron M.G., Ahronheim G., Richard J.E., Riding K., Cron C., Bryer D., MacDonald N., Bouchard M., Young J., Dempsey E.E.** *Comparative efficacies of erythromycin-sulfisoxazole and cefaclor in acute otitis media: a double blind randomized trial.* Pediatr Infect Dis J 1987;6:654-660.

12 **Bergeron M.G., Simard P., Provencher P.** *Influence of growth medium and supplement on growth of Haemophilus influenzae and on antibacterial activity of several antibiotics.* J Clin Microbiol 1987;25:650-655.

13 **Bluestone C.D.** *Management of otitis media in infants and children: current role of old and new antimicrobial agents.* Pediatr Infect Dis J 1988;7:S129-S136.

14 **Burns J.L., Mendelman P.M., Levy J., Stull T.L., Smith A.L.** *A permeability barrier as a mechanism of chloramphenicol resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1985;27:46-54.

15 **Campos J., García-Tornel S., Gairi Tahull J.M.** *Invasive infections caused by multiply resistant Haemophilus influenzae type b.* J Pediatr 1984;104:162-163.

16 **Campos J., García-Tornel S., Sanfeliu I.** *Susceptibility studies of multiply resistant Haemophilus influenzae isolated from pediatric patients and contacts.* Antimicrob Agents Chemother 1984;25:706-709.

17 **Campos J., García-Tornel S., Gairi J.M., Fábregues I.** *Multiply resistant Haemophilus influenzae type b causing meningitis: comparative clinical and laboratory study.* J Pediatr 1986;108:897-902.

18 **Campos J., García-Tornel S.** *Comparative susceptibilities of ampicillin and chloramphenicol resistant Haemophilus influenzae to fifteen antibiotics.* J Antimicrob Chemother 1987;19:297-301.

19 **Campos J., García-Tornel S., Musser J.M., Selander R.K., Smith A.L.** *Molecular epidemiology of multiply resistant Haemophilus influenzae type b in day care centers.* J Infect Dis 1987;156:483-489.

20 **Campos J., Chanyangam M., Groot R. de, Smith A.L., Tenover F.C., Reig R.** *Genetic relatedness of antibiotic resistance determinants in multiply resistant Haemophilus influenzae.* J Infect Dis 1989;160:810-817.

21 **Clymo A.B., Harper I.A.** *Ampicillin-resistant Haemophilus influenzae meningitis.* Lancet 1974;I:453-454.

22 **Cooper T.J., Ladusans E., Williams P.E.O., Polychronopoulos V., Gaya H., Rudd R.M.** *A comparison of oral cefuroxime axetil and oral amoxycillin in lower respiratory tract infections.* J Antimicrob Chemother 1985;16:373-378.

23 **Cox F., Trincher R., Rissing J.P., Patton M., McCracken G.H., Granoff D.M.** *Rifampin prophylaxis for contacts of Haemophilus influenzae type b disease.* JAMA 1981;245:1043-1045.

24 **Dajani A.S., Asmar B.I., Thirumoorthi M.C.** *Systemic Haemophilus influenzae disease: an overview.* J Pediatr 1979;94:355-364.

25 **Dang Van A., Bieth G., Bouanchaud D.H.** *Résistance plasmidique à la tétracycline chez Haemophilus influenzae.* C R Acad Sc Paris 1975;280:1321-1323.

26 **Dang Van A., Goldstein F., Acar J.F., Bouanchaud D.H.** *A transferable kanamycin resistance plasmid isolated from Haemophilus influenzae.* Ann Microbiol 1975;126A:397-399.

27 **Daum R.S., Murphey-Corb M., Shapira E., Dipp S.** *Epidemiology of ROB β-lactamase among ampicillin-resistant Haemophilus influenzae isolates in the United States.* J Infect Dis 1988;157:450-455.

28 **Davies J.E., Smith D.I.** *Plasmid-determined resistance to antimicrobial agents.* Ann Rev Microbiol 1978;32:469-518.

29 **Doern G.V., Chapin K.C.** *Susceptibility of Haemophilus influenzae to amoxicillin/clavulanic acid, erythromycin, cefaclor, and trimethoprim/sulfamethoxazole.* Diagn Microbiol Infect Dis 1986;4:37-41.

30 **Doern G.V., Jorgensen J.H., Thornsberry C., Preston D.A., Haemophilus Influenzae Surveillance Group.** *Prevalence of antimicrobial resistance among clinical isolates of Haemophilus influenzae: a collaborative study.* Diagn Microbiol Infect Dis 1986;4:95-107.

31 **Doern G.V., Daum G.S., Tubert T.A.** *Ampicillin disk diffusion susceptibility testing of Haemophilus influenzae.* J Clin Microbiol 1987;25:1675-1678.

32 **Doern G.V., Jones R.N.** *Antimicrobial susceptibility testing of Haemophilus influenzae, Branhamella catarrhalis, and Neisseria gonorrhoeae.* Antimicrob Agents Chemother 1988;32:1747-1753.

33 **Doern G.V., Jorgensen J.H., Thornsberry C., Preston D.A., Tubert T., Redding J.S., Maher L.A.** *National collaborative study of the prevalence of antimicrobial resistance among clinical isolates of Haemophilus influenzae.* Antimicrob Agents Chemother 1988;32:180-185.

34 **Elwell L.P., Graaff J. de, Selbert D., Falkow S.** *Plasmid-linked ampicillin resistance in Haemophilus influenzae type b.* Infect Immun 1975;12:404-410.

35 **Elwell L.P., Saunders J.R., Richmond M.H., Falkow S.** *Relationships among some R-plasmids found in Haemophilus influenzae.* J Bacteriol 1977;131:356-362.

36 **Engelhard D., Cohen D., Strauss N., Sacks T.G., Jorczak-Sarni L., Shapiro M.** *Randomised study of myringotomy, amoxycillin/clavulanate, or both for acute otitis media in infants.* Lancet 1989;II:141-143.

37 **Eskola J., Peltola H., Takala A.K., Käyhty H., Hakulinen M., Karanko V., Kela E., Rekola P., Rönnberg P.R., Samuelson J.S., Gordon L.K., Mäkelä P.H.** *Efficacy of Haemophilus influenzae type b polysaccharide-diphtheria toxoid conjugate vaccine in infancy.* N Engl J Med 1987;317:717-722.

38 **Feigin R.D., Kline M.W., Spector G.** *Otitis media.* In: Feigin R.D., Cherry J.D. (eds.). Textbook of pediatric infectious diseases, 2nd ed, W.B. Saunders, Philadelphia, 1987;pp 197-215.

39 **Fernandes P.B., Hardy D., Baller R., McDonald E., Pintar J., Ramer N., Swanson R., Gade E.** *Susceptibility testing of macrolide antibiotics against Haemophilus influenzae and correlation of in vitro results with in vivo efficacy in a mouse septicemia model.* Antimicrob Agents Chemother 1987;31:1243-1250.

40 **Fleming D.W., Leibenhaut M.H., Albanes D., Cochi S.L., Hightower A.W., Makintubee S., Helgerson S.D., Broome C.V., Contributing Group** *Secondary Haemophilus influenzae type b in day-care facilities.* JAMA 1985;254:509-514.

41 **Friesen C.A., Cho C.T.** *Characteristic features of neonatal sepsis due to Haemophilus influenzae.* Rev Infect Dis 1986;8:777-780.

42 **Garvey R.J.P., McMullin G.P.** *Meningitis due to β-lactamase producing type b Haemophilus influenzae resistant to chloramphenicol.* Br Med J 1983;287:1183-1184.

43 **Gerber A.C., Pfenninger J.** *Acute epiglottitis management by short duration of intubation and hospitalisation.* Intensive Care Med 1986;12:407-411.

44 **Glode M.P., Daum R.S., Boles E.G., Ballard T.L., Murray M., Granoff D.M.** *Effect of rifampin chemoprophylaxis on carriage eradication and new acquisition of Haemophilus influenzae type b in contacts.* Pediatrics 1985;76:537-542.

45 **Gordon L.K.** *Characterization of a hapten-carrier conjugate vaccine: Haemophilus influenzae-diphtheria conjugate vaccine.* In: Chanock R.M., Lerner R.A. (eds.) Modern approaches to vaccines. Cold Spring Harbor N.Y.: Cold Spring Harbor Press 1984;pp 393-396.

46 **Gordon R.C., Wofford-McQueen R., Shu K.** *In vitro synergism of rifampin-cephalosporin combinations against Haemophilus influenzae type b.* Eur J Clin Microbiol Infect Dis 1990;9:201-205.

47 **Gough J., Kraak W.A.G., Anderson E.C., Nichols W.W., Slack M.P.E., McGhie D.** *Cross-infection by non-encapsulated Haemophilus influenzae.* Lancet 1990;336:159-160.

48 **Graaff J. de, Elwell L.P., Falkow S.** *Molecular nature of two β-lactamase-specifying plasmids isolated from Haemophilus influenzae type b.* J Bacteriol 1976;126:439-446.

49 **Granoff D.M., Basden M.** *Haemophilus influenzae infections in Fresno County, California: a prospective study of the effects of age, race, and contact with a case on incidence of disease.* J Infect Dis 1980;141:40-46.

50 **Granoff D.M., Sheetz K., Pandey J.P., Nahm M.H., Rambeck J.H., Jacobs J.L., Musser J., Selander R.K., Kabeer M., Murphy T.V., Osterholm M.T.** *Host and bacterial factors associated with Haemophilus influenzae type b disease in Minnesota children vaccinated with type b polysaccharide vaccine.* J Infect Dis 1989;159:908-916.

51 **Groot R. de, Campos J., Moseley S.L., Smith A.L.** *Molecular cloning and mechanism of trimethoprim resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1988;32:477-484.

52 **Groot R. de, Chaffin D.O., Kuehn M., Smith A.L.** *Trimethoprim resistance in Haemophilus influenzae is due to altered dihydrofolate reductase(s).* Biochem J 1991;274:657-662.

53 **Harrison L.H., Da Silva G.A., Pittman M., Fleming D.W., Vranjac A., Broome C.V., Brazilian Purpuric Fever Study Group** *Epidemiology and clinical spectrum of Brazilian purpuric fever.* J Clin Microbiol 1989;27:599-604.

54 **Hatch D., Overturf G.D., Kovacs A., Forthal D., Leong C.** *Treatment of bacterial meningitis with ceftazidime.* Pediatr Infect Dis J 1986;5:416-420.

55 **Hedges R.W., Jacob A.E.** *Transposition of ampicillin resistance from RP4 to other replicons.* Mol Gen Genet 1974;132:31-40.

56 **Heffron F., Rubens C.E., Falkow S.** *Translocation of a plasmid DNA sequence which mediates ampicillin resistance: molecular nature and specifity of insertion.* Proc Nat Acad Sci USA 1975;72:3623-3627.

57 **Heffron F., Sublett R., Hedges R.W., Jacob A., Falkow S.** *Origin of the TEM β-lactamase gene found on plasmids.* J Bacteriol 1975;122:250-256.

58 **Howard J.E., Nelson J.D., Clahsen J., Jackson L.H.** *Otitis media of infancy and early childhood.* Am J Dis Child 1976;130:965-970.

59 **Howie V.M., Dillard R., Lawrence B.** *In vivo sensitivity test in otitis media: efficacy of antibiotics.* Pediatrics 1985;75:8-13.

60 **Istre G.R., Conner J.S., Broome C.V., Hightower A., Hopkins R.S.** *Risk factors for primary invasive Haemophilus influenzae disease: increased risk from day care attendance and school-aged household members.* J Pediatr 1985;106:190-195.

61 **Jacobs R.F., Wells T.G., Steele R.W., Yamauchi T.** *A prospective randomized comparison of cefotaxime vs ampicillin and chloramphenicol for bacterial meningitis in children.* J Pediatr 1985;107:129-133.

62 **Jahn G., Laufs R., Kaulfers P.M., Kolenda H.** *Molecular nature of two Haemophilus influenzae R-factors containing resistances and the multiple integration of drug resistance transposons.* J Bacteriol 1979;138:584-597.

63 **Jones R.N.** *Susceptibility testing of Haemophilus influenzae.* Diagn Microbiol Infect Dis 1986;4:93-94.

64 **Jorgensen J.H., Redding J.S., Maher L.A., Howell A.W.** *Improved medium for antimicrobial susceptibility testing of Haemophilus influenzae.* J Clin Microbiol 1987;25:2105-2113.

65 **Juteau J.M., Levesque R.C.** *Sequence analysis and evolutionary perspectives of ROB-1 β-lactamase.* Antimicrob Agents Chemother 1990;34:1354-1359.

66 **Kaulfers P.M., Laufs R., Jahn G.** *Molecular properties of transmissible R-factors of Haemophilus influenzae determining tetracycline resistance.* J Gen Microbiol 1978;105:243-252.

67 **Käythy H., Peltola H., Eskola J., Rönnberg P.R., Kela E., Karanko V., Mäkelä P.H.** *Immunogenicity of Haemophilus influenzae oligosaccharide-protein and polysaccharide-protein conjugate vaccination of children at 4, 6 and 14 months of age.* Pediatrics 1989;84:995-999.

68 **Kemmerich B., Borner K., Pennington J.E.** *Comparative evaluation of enoxacin, ofloxacin, ampicillin, and chloramphenicol for treatment of experimental Haemophilus influenzae pneumonia.* Antimicrob Agents Chemother 1987;31:417-420.

69 **Khan W., Ross S., Rodriguez W.J., Controni G., Saz A.K.** *Haemophilus influenzae type b resistant to ampicillin.* JAMA 1974;229:298-301.

70 **Klingeren B. van, Embden J.D.A. van, Dessens-Kroon M.** *Plasmid-mediated chloramphenicol resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1977;11:383-387.

71 **Krause P.J., Owens N.J., Nightingale C.H., Klimek J.J., Lehmann W.B., Quintiliani R.** *Penetration of amoxicillin, cefaclor, erythromycin-sulfisoxazole, and trimethoprim-sulfamethoxazole into the middle ear fluid of patients with chronic serous otitis media.* J Infect Dis 1982;145:815-821.

72 **Kucers A., Bennett N.McK.** *The use of antibiotics. A comprehensive review with clinical emphasis, 4th edn.* William Heinemann Medical Books, London, 1987.

73 **Kumar A., Kelly K.J.** *In vitro activity of cefixime (CL284635) and other antimicrobial agents against Haemophilus isolates from pediatric patients.* Chemotherapy 1988;34:30-35.

74 **Laufs R., Kaulfers P.M.** *Molecular characterization of a plasmid specifying ampicillin resistance and its relationship to other R-factors from Haemophilus influenzae.* J Gen Microbiol 1977;103:277-286.

75 **Lebel M.H., Hoyt M.J., McCracken G.H.** *Comparative efficacy of ceftriaxone and cefuroxime for treatment of bacterial meningitis.* J Pediatr 1989;114:1049-1054.

76 **Levesque R.C., Medeiros A.A., Jacoby G.A.** *Molecular cloning and DNA homology of plasmid-mediated β-lactamase genes.* Mol Gen Genet 1987;206:252-258.

77 **Levin R.M., Azimi P.H., Dunphy M.G.** *Susceptibility of Haemophilus influenzae type b to cefaclor and influence of inoculum size.* Antimicrob Agents Chemother 1982;22:923-925.

78 **Levy S.B., Buu-Hoi A., Marshall B.** *Transposon Tn10-like tetracycline resistance determinants in Haemophilus parainfluenzae.* J Bacteriol 1984;160:87-94.

79 **Levy J., Burns J.L., Mendelman P.M., Wong K., Mack K.D., Smith A.L.** *Effect of tobramycin on protein synthesis in 2-deoxystreptamine aminoglycoside-resistant clinical isolates of Haemophilus influenzae.* Antimicrob Agents Chemother 1986;29:474-481.

80 **Livrelli V.O., Darfeuille-Richaud A., Rich C.D., Joly B.H., Martel J.L.** *Genetic determinant of the ROB-1 β-lactamase in bovine and porcine Pasteurella strains.* Antimicrob Agents Chemother 1988;32:1282-1284.

81 **Machka K., Braveny I., Dabernat H., Dornbusch K., Dyck E. van, Kayser F.H., Klingeren B. van, Mittermayer H., Perea E., Powell M.** *Distribution and resistance patterns of Haemophilus influenzae: a European cooperative study.* Eur J Clin Microbiol Infect Dis 1988;7:14-24.

82 **Malouin F., Schryvers A.B., Bryan L.E.** *Cloning and expression of genes responsible for altered penicillin-binding proteins 3a and 3b in Haemophilus influenzae.* Antimicrob Agents Chemother 1987;31:286-291.

83 **Manten A., Klingeren B. van, Dessens-Kroon M.** *Chloramphenicol resistance in Haemophilus Influenzae.* Lancet 1976;I:702.

84 **Marburg S., Jorn D., Tolman R.L., Arison B., McCauley J., Knlskern P.J., Hagopian A., Vella P.P.** *Bimolecular chemistry of macromolecules: Synthesis of bacterial polysaccharide conjugates with Neisseria meningitidis membrane protein.* J Am Chem Soc 1986;108:5282-5287.

85 **Marchant C.D., Shurin P.A.** *Antibacterial therapy for acute otitis media: a critical analysis.* Rev Infect Dis 1982;4:506-513.

86 **Marchant C.D., Shurin P.A., Turcyzk V.A., Feinstein J.C., Johnson C.E., Wasikowski D.E., Knapp L.J., Tutlhasi M.A.** *A randomized controlled trial of cefaclor compared with trimethoprim-sulfamethoxazole for treatment of acute otitis media.* J Pediatr 1984;105:633-638.

87 **Marchant C.D., Shurin P.A., Johnson C.E., Murdell-Panek D., Feinstein J.C., Fulton D., Flexon P., Carlin S.A., Van Hare G.F.** *A randomized controlled trial of amoxicillin plus clavulanate compared with cefaclor for treatment of acute otitis media.* J Pediatr 1986;109:891-896.

88 **Markowitz S.M.** *Isolation of an ampicillin-resistant, non-β-lactamase producing strain of Haemophilus influenzae.* Antimicrob Agents Chemother 1980;17:80-83.

89 **Marshall B., Roberts M.C., Smith A.L., Levy S.B.** *Homogeneity of transferable tetracycline resistance determinants in Haemophilus species.* J Infect Dis 1984;149:1028-1029.

90 **McColm A.A., Ryan D.M.** *Therapeutic activity of ceftazidime and eleven other β-lactam antibiotics against experimental Haemophilus influenzae, type b meningitis.* J Antimicrob Chemother 1984;13:517-520.

91 **McCracken G.H., Sakata Y., Olsen K.D.** *Aztreonam therapy in experimental meningitis due to Haemophilus influenzae type b and Escherichia coli K1.* Antimicrob Agents Chemother 1985;27:655-656.

92 **McDougal L.K., Thornsberry C.** *In vitro bactericidal synergism of rifampicin and trimethoprim and implications for treatment of carriers of Haemophilus influenzae.* J Antimicrob Chemother 1982;9:369-378.

93 **McMurry L., Petrucci Jr. R.E., Levy S.B.** *Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in Escherichia coli.* Proc Natl Acad Sci USA 1980;77:3974-3977.

94 **Medeiros A.A., O'Brien T.F.** *Ampicillin-resistant Haemophilus influenzae type b possessing a TEM-type β-lactamase but little permeability barrier to ampicillin.* Lancet 1975;I:716-718.

95 **Medeiros A.A., Levesque R.C., Jacoby G.A.** *An animal source for the ROB-1 β-lactamase of Haemophilus influenzae type b.* Antimicrob Agents Chemother 1986;29:212-215.

96 **Mendelman P.M., Roberts M.C., Smith A.L.** *Mutation frequency of Haemophilus influenzae to rifampicin resistance.* Antimicrob Agents Chemother 1982;22:531-533.

97 **Mendelman P.M., Chaffin D.O., Stull T.L., Rubens C.E., Mack K.D., Smith A.L.** *Characterization of non-β-lactamase-mediated ampicillin resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1984;26:235-244.

98 **Mendelman P.M., Doroshow C.A., Gandy S.L., Syriopoulou V., Welgen C.P., Smith A.L.** *Plasmid-mediated resistance in multiply resistant Haemophilus influenzae type b causing meningitis: molecular characterization of one strain and review of the literature.* J Infect Dis 1984;150:30-37.

99 **Mendelman P.M., Syriopoulou V.P., Gandy S.L., Ward J.I., Smith A.L.** *Molecular epidemiology of plasmid-mediated ampicillin resistance in Haemophilus influenzae type b isolates from Alaska.* J Infect Dis 1985;151:1061-1072.

100 **Mendelman P.M., Chaffin D.O., Clausen C., Stull T.L., Needham C., Williams J.D., Smith A.L.** *Failure to detect ampicillin-resistant, non-β-lactamase producing Haemophilus influenzae by standard disk susceptibility testing.* Antimicrob Agents Chemother 1986;30:274-280.

101 **Mendelman P.M., Smith A.L.** *Haemophilus influenzae.* In: Feigin R.D., Cherry J.D. (eds.). Textbook of pediatric infectious diseases, 2nd edn. W.B. Saunders Company, Philadelphia, 1987;pp 1142-1163.

102 **Mendelman P.M., Chaffin D.O., Musser J.M., Groot R. de, Serfass D.A., Selander R.K.** *Genetic and phenotypic diversity among ampicillin-resistant, non-β-lactamase producing, non-typeable Haemophilus influenzae isolates.* Infect Immun 1987;55:2585-2589.

103 **Mendelman P.M., Henritzy L.L., Chaffin D.O., Lent K., Smith A.L., Stull T.L., Wiley E.A.** *In vitro activities and targets of three cephem antibiotics against Haemophilus influenzae.* Antimicrob Agents Chemother 1989;33:1878-1882.

104 **Mendelman P.M., Wiley E.A., Stull T.L., Clausen C., Chaffin D.O., Önay O.** *Problems with current recommendations for susceptibility testing of Haemophilus influenzae.* Antimicrob Agents Chemother 1990;34:1480-1484.

105 **Mendez B., Tachibana C., Levy S.B.** *Heterogeneity of tetracycline resistance determinants.* Plasmid 1980;3:99-108.

106 **Moxon E.R., Kroll J.S.** *Type b capsular polysaccharide as a virulence factor of Haemophilus influenzae.* Vaccine 1988;6:113-115.

107 **Murphy D., Todd J.** *Treatment of ampicillin-resistant Haemophilus influenzae in soft tissue infections with high doses of ampicillin.* J Pediatr 1979;94:983-987.

108 **Murphy T.V., Osterholm M.T.** *Does rifampin prophylaxis prevent disease caused by Haemophilus influenzae type b?* JAMA 1984;251:2408-2409.

109 **Murphy T.F., Apicella M.A.** *Non-typeable Haemophilus influenzae: a review of clinical aspects, surface antigens, and the human immune response to infection.* Rev Infect Dis 1987;9:1-14.

110 **Murphy T.V., Clements J.F., Breedlove J.A., Hansen E.J., Seibert G.B.** *Risk of subsequent disease among day-care contacts of patients with systemic Haemophilus influenzae type b disease.* N Engl J Med 1987;316:5-10.

111 **National Committee for Clinical Laboratory Standards.** *Performance standards for antimicrobial disk susceptibility tests.* Approved standards M2-A3 National Committee for Clinical Laboratory Standards, Villanova, Pa 1984.

112 **Nelson J.D., Kusmiesz H., Shelton S..** *Cefuroxime therapy for pneumonia in infants and children.* Pediatr Infect Dis J 1982;1:159-163.

113 **Neu H.C.** *New antibiotics: areas of appropriate use.* J Infect Dis 1987;155:403-417.

114 **Nicolle L.E., Postl B., Kotelewetz E., Albritton W.L., Harding G.K.M., Bourgault A.M., Ronald A.R.** *Emergence of rifampin-resistant Haemophilus influenzae.* Antimicrob Agents Chemother 1982;21:498-500.

115 **Odio C.M., Kusmiesz H., Shelton S., Nelson J.D.** *Comparative treatment trial of augmentin versus cefaclor for acute otitis media with effusion.* Pediatrics 1985;75:819-826.

116 **Odio C.M., Faingezicht I., Salas J.L., Guevara J., Mohs E., McCracken G.H.** *Cefotaxime vs. conventional therapy for the treatment of bacterial meningitis of infants and children.* Pediatr Infect Dis J 1986;5:402-407.

117 **Offit P.A., Campos J.M., Plotkin S.A.** *Ampicillin-resistant, β-lactamase-negative Haemophilus influenzae type b.* Pediatrics 1982;69:230-232.

118 **Osterholm M.T., Pierson L.M., White K.E., Libby T.A., Kuritsky J.N., McCullough J.G.** *The risk of subsequent transmission of Haemophilus influenzae type b disease among children in day care.* N Engl J Med 1987;316:1-5.

119 **Parr T.R., Bryan L.E.** *Mechanism of resistance of an ampicillin-resistant, β-lactamase-negative clinical isolate of Haemophilus influenzae type b to β-lactam antibiotics.* Antimicrob Agents Chemother 1984;25:747-753.

120 **Patterson J.E., Madden G.M., Krisiunas E.P., Masecar B., Hierholzer W.J., Zervos M.J., Lyons R.W.** *A nosocomial outbreak of ampicillin-resistant Haemophilus influenzae type b in a geriatric unit.* J Infect Dis 1988;157:1002-1007.

121 **Peltola H., Käyhty H., Virtanen M., Mäkelä P.H.** *Prevention of Haemophilus influenzae type b bacteremic infections with the capsular polysaccharide vaccine.* N Engl J Med 1984;310:1561-1566.

122 **Peltola H., Anttila M., Renkonen O.V., the Finnish study group.** *Randomised comparison of chloramphenicol, ampicillin, cefotaxime and ceftriaxone for childhood bacterial meningitis.* Lancet 1989;I:1281-1287.

123 **Philpott-Howard J., Williams J.D.** *Activity of cephalosporin antibiotics against Haemophilus influenzae.* Scand J Infect Dis 1983;39:S109-S111.

124 **Powell M., Koutsia-Carouzou C., Voutsinas D., Seymour A., Williams J.D.** *Resistance of clinical isolates of Haemophilus influenzae in United Kingdom 1986.* Br Med J 1987;295:176-179.

125 **Powell M., Williams J.D.** *In vitro susceptibility of Haemophilus influenzae to cefixime.* Antimicrob Agents Chemother 1987;31:1841-1842.

126 **Powell M., Williams J.D.** *In vitro activities of aztreonam, imipenem, and amoxycillin-clavulanate against ampicillin-resistant Haemophilus influenzae.* Antimicrob Agents Chemother 1987;31:1871-1873.

127 **Powell M., Livermore D.M.** *Mechanisms of chloramphenicol resistance in Haemophilus influenzae in the United Kingdom.* J Med Microbiol 1988;27:89-93.

128 **Powell M., Williams J.D.** *Detection of ampicillin-resistant Haemophilus influenzae in United Kingdom laboratories.* J Clin Pathol 1988;41:716-719.

129 **Report of the committee on infectious diseases.** American Academy of Pediatrics, 21st edn. Elk Grove Village, ILL 1988.

130 **Ringertz S., Kronvall G.** *Increased use of erythromycin causes resistance in Haemophilus influenzae.* Scand J Infect Dis 1987;19:247-256.

131 **Roberts M.C., Swenson C.D., Owens L.M., Smith A.L.** *Characterization of chloramphenicol-resistant Haemophilus influenzae.* Antimicrob Agents Chemother 1980;18:610-615.

132 **Roberts M.C., Corney A., Shaw W.V.** *Molecular characterization of three chloramphenicol acetyltransferases isolated from Haemophilus influenzae.* J Bacteriol 1982;151:737-741.

133 **Rodriguez W.J., Schwartz R.H., Khan W.N., Gold A.J.** *Erythromycin-sulfisoxazole for persistent acute otitis media due to ampicillin-resistant Haemophilus influenzae.* Pediatr Infect Dis J 1983;2:27-29.

134 **Rodriguez W.J., Schwartz R.H., Salt T., Khan W.N., Chabra O.P., Chang M.J., Reddy S., Marks L.A., Gold A.J.** *Erythromycin-sulfisoxazole vs amoxicillin in the treatment of acute otitis media in children.* Am J Dis Child 1985;139:766-770.

135 **Rodriguez W.J., Puig J.R., Khan W.N., Ferls J., Gold B.G., Sturla C.** *Ceftazidime vs. standard therapy for pediatric meningitis: therapeutic, pharmacologic and epidemiologic observations.* Pediatr Infect Dis J 1986;5:408-415.

136 **Rubin L.G., Medeiros A.A., Yolken R.H., Moxon E.R.** *Ampicillin treatment failure of apparently β-lactamase-negative Haemophilus influenzae type b meningitis due to novel β-lactamase.* Lancet 1981;II:1008-1010.

137 **Sakata Y., McCracken G.H., Thomas M.L., Olsen K.D.** *Pharmacokinetics and therapeutic efficacy of imipenem, ceftazidime, and ceftriaxone in experimental meningitis due to an ampicillin- and chloramphenicol-resistant strain of Haemophilus influenzae type b.* Antimicrob Agents Chemother 1984;25:29-32.

138 **Saunders J.R., Elwell L.P., Falkow S., Sykes R.B., Richmond M.H.** *β-lactamases and R-plasmids of Haemophilus influenzae.* Scand J Infect Dis 1978;13:S16-S22.

139 **Schaad U.B., Suter S., Glanella-Borradori A., Pfenninger J., Auckenthaler R., Bernath O., Cheseaux J-J., Wedgwood J.** *A comparison of ceftriaxone and cefuroxime for the treatment of bacterial meningitis in children.* N Engl J Med 1990;322:141-147.

140 **Schiffer M.S., MacLowry J., Schneerson R., Robbins J.B.** *Clinical, bacteriological, and immunological characterisation of ampicillin-resistant Haemophilus influenzae type b.* Lancet 1974;II:257-259.

141 **Schlamm H.T., Yancovitz S.R.** *Haemophilus influenzae pneumonia in young adults with AIDS, ARC, or risk of AIDS.* Am J Med 1989;86:11-14.

142 **Schleupner C.J., Anthony W.C., Tan J., Flie T.M., Lifland P., Craig W., Vogelman B.** *Blinded comparison of cefuroxime to cefaclor for lower respiratory tract infections.* Arch Intern Med 1988;148:343-348.

143 **Schneerson R., Robbins J.B., Parke J.C., Bell C., Schlesselman J.J., Sutton A., Wang Z., Schiffman G., Karpas A., Shiloach J.** *Quantitative and qualitative analyses of serum antibodies elicited in adults by Haemophilus influenzae type b and Pneumococcus type 6A capsular polysaccharide-tetanus toxoid conjugates.* Infect Immun 1986;52:519-528.

144 **Schwartz R.H., Rodriguez W.J., Khan W.N., Mann R., Barsanti R.G., Ross S.** *Trimethoprim-sulfamethoxazole in the treatment of otitis media caused by ampicillin-resistant strains of Haemophilus influenzae.* Rev Infect Dis 1982;4:514-516.

145 **Seppälä I., Sarvas H., Mäkelä O., Mattila P., Eskola J., Käyhty H.** *Human antibody responses to two conjugate vaccines of Haemophilus influenzae type b saccharides and diptheria toxin.* Scand J Immunol 1988;28:471-479.

146 **Setlow J.K., McCarthy D., Clayton N.L.** *Gene transfer between plasmid and chromosome in Haemophilus influenzae.* In: Pearson M.L., Sternberg N.L. (eds.). Gene transfer and cancer. Raven Press, New York, 1984;pp 15-19.

147 **Shurin P.A., Pelton S.I., Donner A., Finkelstein J., Klein J.O.** *Trimethoprim-sulfamethoxazole compared with ampicillin in the treatment of acute otitis media.* J Pediatr 1980;96:1081-1087.

148 **Simasathien S., Duangmani C., Echeverria P.** *Haemophilus influenzae type b resistant to ampicillin and chloramphenicol in an orphanage in Thailand.* Lancet 1980;II:1214-1217.

149 **Smith A.L.** *Haemophilus influenzae pneumonia.* In: Pennington J.E. (ed.) Respiratory infections: diagnosis and management, 2nd edn. Raven Press, New York, 1988;pp 364-380.

150 **Steinberg E.A., Overturf G.D., Wilkins J., Baraf L.J., Streng J.M., Leedom J.M.** *Failure of cefamandole in treatment of meningitis due to Haemophilus influenzae type b.* J Infect Dis 1978;137:S180-S187.

151 **Strandberg D.A., Jorgensen J.H., Drutz D.J.** *Activities of newer β-lactam antibiotics against ampicillin, chloramphenicol, or multiply-resistant Haemophilus influenzae.* Diagn Microbiol Infect Dis 1984;2:333-337.

152 **Sturm A.W., Mostert R., Rouing P.J.E., Klingeren B. van, Alphen L. van.** *Outbreak of multi-resistant non-encapsulated Haemophilus influenzae infections in a pulmonary rehabilitation centre.* Lancet 1990;335:214-216.

153 **Stuy J.H.** *Plasmid transfer in Haemophilus influenzae.* J Bacteriol 1979;139:520-529.

154 **Swaminathan B., Mayer L.W., Bibb W.F., Ajello G.W., Irino K., Birkness K.A., Garon C.F., Reeves M.W., De Cunto Brandileone M.C., Sottnek F.O., Brenner D.J., Steigerwalt A.G., Brazilian Purpuric Fever Study Group.** *Microbiology of Brazilian purpuric fever and diagnostic tests.* J Clin Microbiol 1989;27:605-608.

155 **Sydnor A., Gwaltney J.M., Cocchetto D.M., Scheld W.M.** *Comparative evaluation of cefuroxime axetil and cefaclor for treatment of acute bacterial maxillary sinusitis.* Arch Otolaryngol Head Neck Surg 1989;115:1430-1433.

156 **Syriopoulou V., Scheifele D., Smith A.L., Perry P.M., Howie V.** *Increasing incidence of ampicillin resistance in Haemophilus influenzae.* J Pediatr 1978;92:889-892.

157 **Takala A.K.** *Epidemiologic characteristics and risk factors for invasive Haemophilus influenzae type b disease in a population with high vaccine efficacy.* Pediatr Infect Dis J 1989;8:343-346.

158 **Takala A.K., Eskola J., Palmgren J., Rönnberg P.R., Kela E., Rekola P., Mäkelä P.H.** *Risk factors of invasive Haemophilus influenzae type b disease among children in Finland.* J Pediatr 1989;115:694-701.

159 **Thomas W.J., McReynolds J.W., Mock C.R., Balley D.W.** *Ampicillin-resistant Haemophilus influenzae meningitis.* Lancet 1974;I:313.

160 **Thornsberry C., Kirven L.A.** *Ampicillin resistance in Haemophilus influenzae as determined by a rapid test for β-lactamase production.* Antimicrob Agents Chemother 1974;6:653-654.

161 **Tomeh M.O., Starr S.E., McGowan J.E., Terry P.M., Nahmias A.J.** *Ampicillin-resistant Haemophilus influenzae type b infection.* JAMA 1974;229:295-297.

162 **Uchiyama N., Greene G.R., Kitts D.B., Thrupp L.D.** *Meningitis due to Haemophilus influenzae type b resistant to ampicillin and chloramphenicol.* J Pediatr 1980;97:421-424.

163 **Umetsu D.T., Ambrosino D.M., Quinti I., Siber G.R., Geha R.S.** *Recurrent sinopulmonary infection and impaired antibody response to bacterial capsular polysaccharide antigen in children with selective IgG-subclass deficiency.* N Engl J Med 1985;313:1247-1251.

164 **Wallace R.J., Baker C.J., Quinones F.J., Hollis D.G., Weaver R.E., Wiss K.** *Non-typeable Haemophilus influenzae (biotype 4) as a neonatal, maternal and genital pathogen.* Rev Infect Dis 1983;5:123-136.

165 **Wallace R.J., Steele L.C., Brooks D.L., Luman J.I., Wilson R.W., McLarty J.W.** *Amoxicillin-clavulanic acid in the treatment of lower respiratory tract infections caused by β-lactamase-positive Haemophilus influenzae and Branhamella catarrhalis.* Antimicrob Agents Chemother 1985;27:912-915.

166 **Wallace R.J., Steele L.C., Brooks D.L., Forrester G.D., García J.G.N., Luman J.I., Wilson R.W., Shepherd S., McLarty J.** *Ampicillin, tetracycline, and chloramphenicol resistant Haemophilus influenzae in adults with chronic lung disease.* Am Rev Respir Dis 1988;137:695-699.

167 **Weinberg G.A., Ghafoor A., Ishaq Z., Nomani N.K., Kabeer M., Anwar F., Burney M.I., Qureshi A.W., Musser J.M., Selander R.K., Granoff D.M.** *Clonal analysis of Haemophilus influenzae isolated from children from Pakistan with lower respiratory tract infections.* J Infect Dis 1989;160:634-643.

168 **Willard J.E., Johnson E.J., Daum R.S.** *Interrelationships between physical state, phenotypic stability and transferability of β-lactamase genes in Haemophilus influenzae.* J Gen Microbiol 1982;128:2353-2360.

169 **Wolfson J.S., Hooper D.C.** *Bacterial resistance to quinolones: mechanisms and clinical importance.* Rev Infect Dis 1989;11:S960-S968.

170 **Wong V.K., Wright H.T., Mason W.H., Ross L.A., Inderlied C.B., Kim K.S.** *Use of imipenem-cilastatin in pediatric patients with bacterial meningitis.* In: Program and abstracts of the twenty-eighth interscience conference on antimicrobial agents and chemotherapy. Washington DC: American Society for Microbiology 1988;112.

171 **Yogev R., Moxon E.R.** *Elaboration of type b capsule by Haemophilus influenzae as a determinant of pathogenicity and impaired killing by trimethoprim-sulfamethoxazole.* J Clin Invest 1982;69:658-665.

CHAPTER 5

MOLECULAR CLONING AND MECHANISM OF TRIMETHOPRIM RESISTANCE IN HAEMOPHILUS INFLUENZAE

Antimicrob Agents Chemother 1988;32:477-484.

CHAPTER 5

MOLECULAR CLONING AND MECHANISM OF TRIMETHOPRIM RESISTANCE IN HAEMOPHILUS INFLUENZAE

Ronald de Groot[1,2], José Campos[1], Steve L. Moseley[2,3] and Arnold L. Smith[1,2]

[1] *Division of Infectious Disease, Children's Hospital and Medical Center, Seattle, USA.*
[2] *Department of Pediatrics, University of Washington, Seattle, USA.*
[3] *Department of Microbiology, University of Washington, Seattle, USA.*

5.1 SUMMARY

We studied 10 trimethoprim-resistant (Tmp^r) *Haemophilus influenzae* isolates for which agar dilution MICs were 10 to > 200 μg/ml. Trimethoprim resistance was transferred from two Tmp^r *H. influenzae* isolates to a Tmp^s strain by conjugation or transformation. Wild-type Tmp^r strains and Tmp^r transcipients did not contain detectable plasmid DNA. The trimethoprim resistance gene was cloned into a cosmid vector, and recombinant plasmids were transduced into *Escherichia coli*. A 0.50-kilobase intragenic probe derived from a 12.9-kilobase fragment which encoded trimethoprim resistance hybridized with whole-cell DNA from Tmp^s and Tmp^r strains. Southern blot analysis of restricted DNA from isogenic Tmp^s and Tmp^r *H. influenzae* indicated that acquisition of trimethoprim resistance involved a rearrangement or change in nucleotide sequence. Hybridization was not seen with DNA derived from Tmp^r *E. coli* containing dihydrofolate reductase I, II, and III genes or with Tmp^r *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Pseudomonas cepacea*. Southern hybridization with 12 multiple-resistant encapsulated *H. influenzae* strains confirmed that the trimethoprim resistance gene was chromosomally mediated. Dihydrofolate reductase activity was significantly greater in cell sonicate supernatants of Tmp^r strains in comparison with isogenic Tmp^s recipients. Differences were not found in the trimethoprim inhibition profile of dihydrofolate reductase activity in Tmp^s and Tmp^r strains.

We conclude that the mechanism of trimethoprim resistance in *H. influenzae* is overproduction of chromosomally located dihydrofolate reductase.

5.2 INTRODUCTION

Haemophilus influenzae is an important cause of infections, especially in children. Unencapsulated *H. influenzae* is a major cause of otitis media, sinusitis, and conjunctivitis, whereas *H. influenzae* type b is frequently isolated in invasive disease. Trimethoprim-sulfamethoxazole is recommended as an effective alternate therapy to ampicillin for otitis media and sinusitis [15, 49] and is occasionally used for the treatment of invasive disease caused by *H. influenzae*. Trimethoprim interferes with the synthesis of tetrahydrofolate by inhibition of the enzyme dihydrofolate reductase (DHFR): the subsequent methylation of uridine to yield thymidine is inhibited. A survey of antibiotic resistance in *H. influenzae* in England found that 1.4% of the isolates were Tmp^r (MIC > 4 μg/ml) [42]. A higher incidence of trimethoprim resistance in multiple-resistant encapsulated *H. influenzae* was found in Spain [10]. Data on the mechanism of trimethoprim resistance in *H. influenzae* are not available. At least seven mechanisms for trimethoprim resistance have been described in other genera. Intrinsic resistance can be caused by decreased cell wall permeability [59] or can be due to the production of a trimethoprim-resistant DHFR [56]. Mutational resistance has been attributed to four different mechanisms: thymine auxotrophy [33], overproduction of DHFR [16], production of an altered DHFR with decreased affinity for trimethoprim [51], and decreased outer membrane permeability [21]. In addition, R-plasmid-specified resistance has been described [2, 3, 19, 41, 55].
We studied 10 Tmp^r *H. influenzae* strains, testing them by agar dilution on a thymidine-free medium [111]. We investigated the mechanism of resistance in unencapsulated strains and cloned the gene responsible for trimethoprim resistance. The trimethoprim resistance gene in *H. influenzae* was compared with the trimethoprim resistance gene in other micro-organisms.

5.3 MATERIALS AND METHODS

Bacterial strains

The clinical *H. influenzae* strains used in this study are listed in Table 5.1. Laboratory strains are listed in Table 5.2. All isolates of *H. influenzae* were characterized by an obligate growth requirement for hemin and β-NAD^+ when incubated at 37°C in air and by morphology. All strains were typed by slide agglutination with polyvalent and type b sera

Table 5.1 Description of *H. influenzae* isolates.

Strain	Relevant characteristics[a]	Geographic origin	Isolation site	Date isolated	Reference or source
R648	Tmp^r Amp^r Cap^-	Germany	Sputum	1980	[7]
R721	Tmp^r Cap^-	Sweden	Sputum	1979	[61]
R722	Tmp^r Amp^r Cap^-	Sweden	Abscess	1979	[61]
R846	Tmp^r Amp^r Cap^-	England	NA[b]	1980	N. Datta
R1037	Tmp^r Cap^-	England	Eye	1981	[42]
R1040	Tmp^r Cap^-	England	Eye	1981	[42]
R1041	Tmp^r Cap^-	England	Sputum	1981	[42]
R1042	Tmp^r Cap^-	England	Sputum	1981	[42]
R1043	Tmp^r Cap^-	England	Eye	1981	[42]
R1047	Tmp^r Cap^-	England	Eye	1981	[42]
R1661, R1664	Tmp^r Amp^r Cm^r Tc^r Cap^+	Spain	Cerebrospinal fluid	1981-1983	[9]
R1680, R1696	Tmp^r Amp^r Cm^r Tc^r Cap^+	Spain	Cerebrospinal fluid	1981-1983	[9]
R1707, R1708	Tmp^r Amp^r Cm^r Tc^r Cap^+	Spain	Cerebrospinal fluid	1981-1983	[9]
R1715, R1716	Tmp^r Amp^r Cm^r Tc^r Cap^+	Spain	Cerebrospinal fluid	1981-1983	[9]
R1728, R1730	Tmp^r Amp^r Cm^r Tc^r Cap^+	Spain	Cerebrospinal fluid	1981-1983	[9]

[a] Abbreviations: Amp, ampicillin; Cap, capsule; Cm, chloramphenicol; r, resistant; Rif, rifampin; s, susceptible; Tc, tetracycline; Tmp, trimethoprim.
[b] NA, Not available.

(Difco Laboratories, Detroit, Mich.). Twenty-seven strains of *Neisseria meningitidis* for which MICs of penicillin G were between 0.03 and 0.7 μg/ml and MICs of trimethoprim were between 10 and 200 μg/ml were also studied. Eight Tmp^r *Neisseria gonorrhoeae* strains (JSK11, JSK269, A174, K1944, P1, F18, F28 and F29) (MICs of trimethoprim were between 10 and 100 μg/ml) were kindly provided by Rosalyn Rice (University of Washington, Seattle). *Pseudomonas cepacea* 725 RN (MIC of trimethoprim, 5 μg/ml), PC 138 (MIC of trimethoprim, 20 μg/ml), and PC 178 (MIC of trimethoprim, 200 μg/ml) were kindly provided by Jane L. Burns (University of Washington, Seattle).

Media

The medium for growth of *H. influenzae* was brain heart infusion agar or broth (Difco) supplemented with 10 μg of hemin chloride per ml, 10 μg of histidine per ml, and 2 μg of β-NAD^+ per ml (sBHI). Plates were incubated overnight at 36.5°C in 5% CO_2. Liquid cultures were incubated at 37°C in air and shaken at 150 rpm. The MIC of trimethoprim was determined on Catlin medium [11] that was solidified with 2.5% agarose obtained

Table 5.2 Laboratory strains and plasmids.

Strain	Relevant characteristics[a]	Reference or source
Haemophilus influenzae		
R906	Ery^r Sm^r $Spec^r$ Rif^s Cap^-	[12]
Goodgal	Rec^+ Leu^- Met^- Hyp^- Lys^- Val^- Ala^-	[38]
Rec1	Rec^-, no antibiotic resistance markers	[48]
Rec1Rif	Rec1 selected for spontaneous Rif^r	[36]
E1a	Sm^r Cap^+	[50]
RSF007	Amp^r, contains 30-megadalton plasmid	[47]
R842	Amp^r, contains 4.1-kb plasmid	[13]
R906/1042-1	Tmp^r Sm^r Ery^r transcipient of R906 and R1042	
R906/1047-1	Tmp^r Sm^r Ery^r transcipient of R906 and R1047	
Escherichia coli		
HB101	F^- *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44 leu*$^-$ *thi*$^-$	[5, 6]
P700	K-12, C600(pUC4-12) Tmp^r, contains the gene for type II DHFR	[17]
P872	K-12, C600 Tmp^r, contains the type I DHFR structural gene	[18]
P1242	K-12, JM83 Tmp^r, contains the gene for type III DHFR	M.E. Fling
pHC79	Amp^r Tc^r Cos region of lambda	[24]
pGEM3	Amp^r recombinant plasmid containing a pUC18-derived multiple cloning region	[35]

[a] Abbreviations: Amp, ampicillin; Cap, capsule; Cm, chloramphenicol; Ery, erythromycin; r, resistant; Rec, recombination proficiency; Rif, rifampin; s, susceptible; Spec, spectinomycin; Sm, streptomycin; Tc, tetracycline; Tmp, trimethoprim.

from Sigma Chemical Co. (St.Louis, Mo.). LB broth [31] and Mueller-Hinton agar (Difco) were used to grow *Escherichia coli*. Gonococcal, meningococcal, and *P. cepacea* strains were grown on chocolate agar supplemented with GC base (Difco) containing 5% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). Gonococcal strains were incubated for 18 h at 36.5°C in a candle jar. Meningococci were incubated overnight at 36.5°C in 5% CO_2 and *E. coli* strains were incubated overnight at 36.5°C in air.

Antibiotics

Trimethoprim was obtained from Sigma. The antibiotic stock solution used for determination of the MIC was 5 mg/ml (17.24 mM) in 50 mM HCl. Ampicillin, erythromycin, rifampin, and streptomycin were also purchased from Sigma.

Other materials

Restriction enzymes and T4 DNA ligase were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). DHFR, dihydrofolic acid, alkaline phosphatase, and β-NADPH were obtained from Sigma. Nucleotides ([α-^{32}P]dATP, [α-^{32}P]dCTP, dTTP and dGTP) were obtained from New England Nuclear Corp. (Boston, Mass.). Cesium chloride was purchased from Gallard Schlesinger Chemical Manufacturing Corp. (New York, N.Y.). Agarose was obtained from Seakem Corp. (Rockport, Maine).

Antimicrobial susceptibility testing

Determinations of the MIC of trimethoprim for 14 *H. influenzae* strains were performed by agar dilution [29]. Bacteria were inoculated with a Steers replicator on Catlin medium [11] solidified with 2.5% agarose (Catlin agarose) containing trimethoprim at graded concentrations between 0.1 and 200 μg/ml. The inocula tested were 10^5, 10^3, and 10^1 CFU. The MIC was defined as the lowest concentration that completely inhibited visible growth in comparison with growth on antibiotic-free medium.

Conjugation

Matings between Tmp^r wild-type strains and Tmp^s recipients (R906, Rec1Rif) were performed as described previously [47]. After overnight incubation on micropore filters, the cells were inoculated in duplicate on Catlin agarose containing 10 μg of trimethoprim per ml and 250 μg of streptomycin per ml. The conjugation frequency was determined by dividing the number of transcipients by the number of donor cells plated. The frequency of mutation to trimethoprim resistance was determined by plating 10^9 bacteria on Catlin agarose containing trimethoprim (10 μg/ml). The frequency of mutation to rifampin resistance was determined by plating 10^9 bacteria on sBHI agar containing rifampin (10 μg/ml). The concentration of trimethoprim and streptomycin inhibiting the growth of transcipients was determined by the agar dilution technique. Transcipients from matings between R906 and R1042 and between R906 and R1047 were subsequently mated with Rec1Rif. Cells from these matings were inoculated in duplicate on Catlin agarose containing trimethoprim at 20 μg/ml and rifampin at 10 μg/ml.

Transformation to trimethoprim resistance with total DNA

Transformations were performed with *H. influenzae* Goodgal and Rec1 as the recipients. The bacterial cells were made competent by the procedure of Herriott et al. [23]. Whole-cell lysates of trimethoprim-resistant transcipients R906/1042-1 and R906/1047-1 (Table 5.1) were prepared as described previously [46]. Approximately 100 to 1,000 ng of DNA

150 rpm). After 4 ml of sBHI broth was added, cells were incubated for two additional hours at 37°C. Micro-organisms were then plated on appropriate antibiotic media and on sBHI plates to determine bacterial density and verify the lack of contamination. The antibiotic resistance of transformants was confirmed by growth on Catlin agarose containing trimethoprim at 10 μg/ml. Colonies were also inoculated on sBHI agar plates with streptomycin at 250 μg/ml and erythromycin at 20 μg/ml. The transformation frequency to trimethoprim resistance was determined by the number of Tmp^r transformant colonies divided by the total number of cells plated.

Plasmids

Strains R906, R1042, and R1047 and 16 transcipient strains derived from matings between R906 and R1042, R906 and R1047, Rec1Rif and transcipient R906/1042-1, and Rec1Rif and transcipient R906/1047-1 were examined for the presence of plasmids by five different techniques [22, 26, 27, 37, 43]. Reference Tmp^s *H. influenzae* strains containing plasmids were R842 [13] and RSF007 [47]. Large-scale plasmid isolations were performed by the method of So et al.[52].

Isolation of total DNA from *H. influenzae*

Isolation of whole-cell DNA was performed by the method of Hull et al. [25]. Whole-cell DNA was isolated by centrifugation in cesium chloride and dialyzed extensively against TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Dialysis tubing was prepared as described previously [31].

Nucleic acid techniques

Restriction enzymes were used according to the guidelines of the manufacturer, with buffers prepared as described previously [31]. Plasmid and chromosomal DNAs were electrophoresed in 0.3 to 1.5% agarose gels. Agarose with a low gelling temperature (FMC Corp., Marine Colloids Div., Rockland, Maine) was used to isolate DNA fragments of interest. Ligations, transformations of competent *E. coli* HB101, and dephosphorylation of vector DNA with calf intestinal alkaline phosphatase were performed by standard methods [31]. Gels were stained with ethidium bromide, visualized by UV transillumination, and photographed with a Polaroid M4 camera. Colony hybridizations were performed as described previously [31]. Transfer of DNA from agarose gels to Nytran paper (0.45-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) was performed as previously described [53]. Hybridizations were performed under stringent conditions. Briefly, filters were prehybridized for 1 h at 42°C in a buffer containing 50% formamide, 100 μg of sonicated salmon sperm DNA per ml, 5 x Denhardt solution (1 x Denhardt solution is 0.02%

polyvinylpyrrolidone, 0.02% bovine serum albumin, and 0.02% Ficoll), and 5 x SSC buffer (1 x SSC buffer contains 0.15 M sodium chloride plus 0.015 M sodium citrate). The solution was discarded and replaced with the same buffer containing the probe. Hybridization was done at 42°C overnight. Filters were washed at room temperature in 2 x SSC-0.1% sodium dodecyl sulfate for 15 min and then for an additional 15 min in the same buffer at 65°C. Autoradiography was performed by the method of Wahl et al. [58].

Preparation of cosmid library and screening for trimethoprim gene

A partial digestion of whole-cell DNA of strain R1047 was performed with *Sau*3AI. The DNA was banded by gradient centrifugation in cesium chloride, and fragments between 35 and 45 kilobases (kb) were selected. *Sau*3AI-digested DNA (9 μg) was ligated to 2 μg of pHC79 digested with *Bam*HI in a volume of 90 μl. The ligated DNA was packaged with an in vitro packaging mixture obtained from Vector Cloning Systems (San Diego, Ca.) [24]. Recombinant cosmids were transduced into *E. coli* HB101, selecting on ampicillin (100 μg/ml) and trimethoprim (5 μg/ml). The ampicillin and trimethoprim resistance phenotype was confirmed on Catlin agarose plates containing 100 μg of ampicillin per ml and 5 μg of trimethoprim per ml. In selected recombinants, the presence of plasmid and insert was confirmed by electrophoresis in agarose as described previously [4]. Plasmid DNA from six recombinant clones prepared by the method of Birnboim [4] was tested for its ability to transform strain Goodgal to trimethoprim resistance. *Eco*RI-digested pHC79 DNA was used as a negative control.

Subcloning of trimethoprim resistance gene and construction of restriction enzyme map

Plasmid DNA of pRGS1 (a Tmp^r recombinant cosmid) was digested with *Eco*RI and electrophoresed. After staining and visualization, nine bands (ranging in size from 12.9 to < 1 kb) were visualized and excised and the DNA was electroeluted. Each fragment was tested for its ability to transform competent strain Goodgal to trimethoprim resistance. The 12.9-kb fragment (125 ng), which appeared to contain the trimethoprim resistance gene, was ligated to 67 ng of dephosphorylated *Eco*RI-digested pGEM3 [35] in a volume of 10 μl. The ligation mixture was transformed into *E. coli* HB101, and recombinants were selected on ampicillin (50 μg/ml) and trimethoprim (5 μg/ml). The presence of a 12.9-kb insert coding for trimethoprim resistance was confirmed by electrophoresis in agarose. The DNA of this subclone (pRGS7) was digested with the following restriction enzymes: *Acc*I, *Ava*I, *Bam*HI, *Bgl*I, *Dde*I, *Eco*RI, *Hin*cII, *Hin*dIII, *Hin*fI, *Hpa*II, *Pst*I, *Sal*I, *Sph*I, *Xba*I, and *Xho*I. Single and double digestions with restriction enzymes were performed to obtain a restriction enzyme map. pRGS7 DNA was digested with each of the above enzymes, and the resulting fragments were used to transform strain Goodgal to trimethoprim resistance.

Construction of hybridization probe

A 0.50-kb *Xba*I-*Pst*I fragment from pRGS7 was excised after electrophoresis on low-melting-temperature agarose. The DNA was electroeluted, extracted with phenol-chloroform, and ethanol precipitated. This fragment was used as a probe in colony blots and Southern hybridizations. This fragment was also tested for its ability to transform competent strain Goodgal to trimethoprim resistance; whole-cell DNA derived from pRGS7, a 2.2-kb *Hin*fI fragment of pRGS7, and distilled water were used as controls.
The 0.50-kb DNA fragment was labeled by nick translation with [α-^{32}P]dCTP and [α-^{32}P]dATP as described previously [45]. The unincorporated nucleotides were separated from the probe with a Gene Clean kit (Bio 101, Inc., La Jolla, Ca.). DNA-DNA hybridizations were performed under stringent conditions [31].

Preparation of crude enzyme extracts

Crude enzyme extracts were prepared as described previously [55]. After suspension of the cells in buffer (0.05 M Tris chloride [pH 7.2], 1mM dithiothreitol, 0.05 M potassium chloride, 1 mM sodium EDTA), the bacteria were sonicated with six cycles of 20 s duration, at 20 W in a W370 cell disrupter (Heat Systems Ultrasonics) in an ice bath. After centrifugation of the sonicate at 10,000 x g for 15 min, the supernatants were stored at -20°C. The protein concentration of the cell extracts was measured as described before [30].

DHFR assay

DHFR activity was measured spectrophotometrically as described previously [40]. The assay was performed with a Beckman model DU 6 spectrophotometer. Enzyme activity was detected by measuring the decrease in A_{340} with time at a temperature of 30°C, using cell sonicate supernatants. Samples of cell extracts were incubated for 3 min at 30°C with NADPH (0.1 mmol) with or without trimethoprim in concentrations ranging from 10^{-3} to 10^{-8} M. Dihydrofolic acid (0.1 mmol) was then added, and the decrease in absorbance was measured during the initial 30 tot 60 s to estimate the initial velocity. We defined 50% inhibition as the molar concentration of trimethoprim necessary to inhibit 50% of enzyme activity.

Outer membrane proteins

Outer membrane proteins were isolated by the lithium chloride extraction technique [34]. Protein concentration of the samples was measured as described previously [30]. Samples of 5 μg were electrophoresed on 8% polyacrylamide gels prepared as described

previously [31]. Protein bands were identified by the silver staining technique of Wray et al. [60].

5.4 RESULTS

MIC

The MIC of trimethoprim for the trimethoprim-resistant *H. influenzae* clinical isolates varied from 10 to > 200 μg/ml (Table 5.3). Susceptible strains were defined as those whose growth was inhibited by trimethoprim at ≤ 0.5 μg/ml. With the exception of R906 and E1a, all strains were susceptible to streptomycin (MIC, ≤ 25 μg/ml). There was a slight inoculum effect with the trimethoprim-resistant strains. For example, the MIC of trimethoprim for strain R1047 at 10^1 CFU was 5 μg/ml, at 10^3 CFU it was 10 μg/ml, and at 10^5 CFU it was 40 μg/ml. This phenomenon was not seen with trimethoprim-susceptible cells.

Conjugation

Trimethoprim-resistant strains R1042 and R1047 were mated separately with laboratory strain R906. Transcipients from these matings were used in additional mating experiments to retransfer resistance to strain Rec1Rif. Trimethoprim resistance was transferred at a frequency ranging from 10^{-5} to 10^{-6} per donor cell. The spontaneous mutation frequency to trimethoprim resistance was < 10^{-9} with all recipients. The spontaneous mutation frequency of R906/1042-1 and R906/1047-1 to rifampin resistance was 10^{-9}. Transcipients R906/1042-1 and R906/1047-1 were trimethoprim-, streptomycin-, and erythromycin-resistant. Isolates derived from matings with strain Rec1Rif were trimethoprim- and rifampin-resistant. However, 90% of 88 Tmp^r transcipients obtained with Rec1Rif were streptomycin-resistant and 61% were erythromycin-resistant.

Plasmid DNA detection

Using different plasmid isolation techniques, we were unable to detect plasmid DNA in two trimethoprim-resistant wild strains (R1042 and R1047) and in 16 trimethoprim-resistant transcipients. We were, however, successful in visualizing plasmids in the strains RSF007 and R842.

Transformation

DNA derived from transcipient strains R906/1042-1 and R906/1047-1 transferred trimethoprim resistance to *H. influenzae* Goodgal (recombination proficient) by transformation at a

Table 5.3 Antibiotic susceptibility.

Strain	MIC of trimethoprim (μg/ml)[a]	MIC of streptomycin (μg/ml)
R648	10	5
R721	100	5
R722	40	5
R846	10	5
R906	0.5	> 250
R1037	40	5
R1040	40	25
R1041	40	25
R1042	> 200	25
R1043	20	25
R1047	40	5
E1a	0.5	> 250
Rec1	0.5	5
Goodgal	0.5	5
Rec1Rif	0.5	5
HB101	0.5	> 250

[a] Determined by agar dilution with an inoculum of 10^5 CFU.

frequency of 1 x 10^{-3} to 2 x 10^{-3}. Transfer of resistance to *H. influenzae* Rec1 occurred at a much lower frequency (1.5 x 10^{-6} to 1 x 10^{-7}). Fifty transformant colonies derived from strain Goodgal grew on trimethoprim at 10 μg/ml, but did not grow on erythromycin at 20 μg/ml. Of 50 transformants, 1 was resistant to streptomycin at 250 μg/ml; the other 49 did not grow on streptomycin at 250 μg/ml.

Cloning of trimethoprim resistance gene

A cosmid library was prepared from the trimethoprim-resistant strain R1047 and screened in *E. coli* HB101. Recombinant clones were selected on L agar containing ampicillin (100 μg/ml) and trimethoprim (5 μg/ml).

The phenotype (Tmp^r Amp^r) of six recombinant cosmid clones (pRGS1 to pRGS6) was confirmed, and the presence of inserts was shown by an increase in the apparent plasmid mass relative to pHC79. Transformations of competent strain Goodgal with plasmid DNA from these clones yielded Tmp^r transformants at high frequencies (10^{-3}). Transformation with EcoR1-digested pHC79 as a control did not yield Tmp^r strains (frequency < 10^{-9}). A 12.9-kb EcoR1 fragment derived from pRGS1 transformed strain

Goodgal to trimethoprim resistance at high frequency ($\sim 10^{-3}$). This fragment was ligated into pGEM3, and the ligation mixture was transformed into HB101. The recombinant plasmid (pRGS7) was confirmed to confer trimethoprim resistance.

Construction of restriction enzyme map

Plasmid pRGS7 containing the trimethoprim resistance gene was subjected to restriction analysis, and a map was constructed (Figure 5.1). Transformation to trimethoprim resistance was not possible with *Pst*I- or *Xba*I-digested fragments, indicating an intragenic site for both enzymes. Transformation to Tmp^r after digestion with the other enzymes listed in Figure 5.1 was observed at high frequency (10^{-4}). Transformation of strain Goodgal to Tmp^r with a 0.50-kb *Xba*I-*Pst*I fragment of pRGS7 (Figure 5.1) was not detected, whereas transformation with a 2.2-kb *Hin*fI fragment which contained the 0.50-kb fragment produced Tmp^r colonies at a frequency of 10^{-4}. These data indicate that the 0.50-kb *Xba*I-*Pst*I fragment is intragenic.

Screening for trimethoprim resistance by colony hybridization

On colony blots, the 0.50-kb *Pst*I-*Xba*I probe hybridized to all unencapsulated trimethoprim-resistant *H. influenzae* strains shown in Table 5.1 and to transcipients R906/1042-1 and R906/1047-1. However, the probe also hybridized with trimethoprim-susceptible strains R906 (unencapsulated) and E1a (encapsulated). Colony blots with 10 multiple-resistant encapsulated *H. influenzae* strains from Spain and 10 Tmp^s Amp^r Cm^r Tc^r transcipients in R906 all produced a prominent signal. Hybridization of the probe with

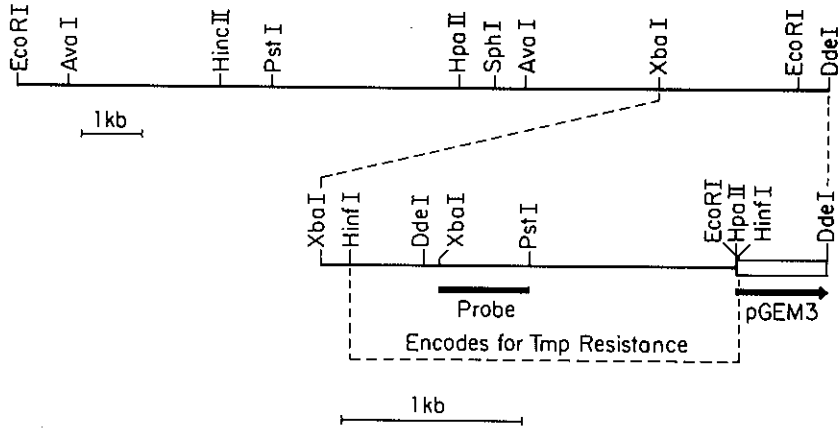

Figure 5.1 Restriction endonuclease map of pRGS7. The remaining 2.4-kb fragment from pGEM3 is not included.

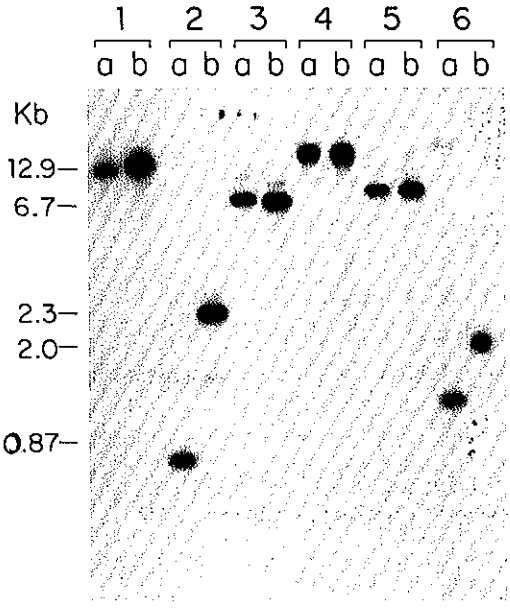

Figure 5.2 Southern analysis of whole-cell DNA restriction enzyme digests from trimethoprim-susceptible (R906) (lanes a) and trimethoprim-resistant (R906/1047-1) (lanes b) isogenic *H. influenzae* strains. The fragments were separated by agarose gel electrophoresis, transferred onto Nytran paper, and probed with ^{32}P-labeled *Xba*I-*Pst*I probe (500 base pairs). Lanes represent restriction enzyme digests: 1, *Eco*RI; 2, *Dde*I; 3, *Hpa*II; 4, *Xba*I; 5, *Pst*I; 6, *Hin*fI.

E. coli P700, P872, and P1242 was not detected, indicating the absence of homology between the type I, II, and III DHFR genes in *E. coli* and the trimethoprim resistance gene in *H. influenzae*. The probe hybridized with *E. coli* HB101 (pRGS2) and HB101 (pRGS3) (both containing the trimethoprim resistance gene from R1047 cloned into pHC79) and with *E. coli* HB101 (pRGS7). Colony hybridizations with the 0.5-kb probe with *E. coli* HB101 containing pGEM3 and with *E. coli* HB101 did not reveal homology. Hybridization with lysed colonies of 8 *N. gonorrhoeae* strains, 27 *N. meningitidis* strains, and 3 *P. cepacea* strains with various levels of trimethoprim resistance did not reveal homology.

Southern blot analysis

Colony hybridizations with Tmp^r and Tmp^s *H. influenzae* suggested homology between the cloned gene encoding trimethoprim-susceptible strains. We performed Southern blot analysis of DNA from isogenic trimethoprim-susceptible and trimethoprim-resistant strains (R906 and R906/1047-1). Total DNA from each strain was digested with different restriction enzymes and then hybridized with the 0.50-kb probe (Figure 5.2). Digestions with *Dde*I and *Hin*fI showed hybridization with fragments of different molecular size in the two strains. To confirm that trimethoprim resistance in *H. influenzae* is encoded chromosomally, we performed Southern blot analysis on agarose gels with separated plasmid and chromosomal DNA from four multiple-resistant *H. influenzae* strains from Spain and four

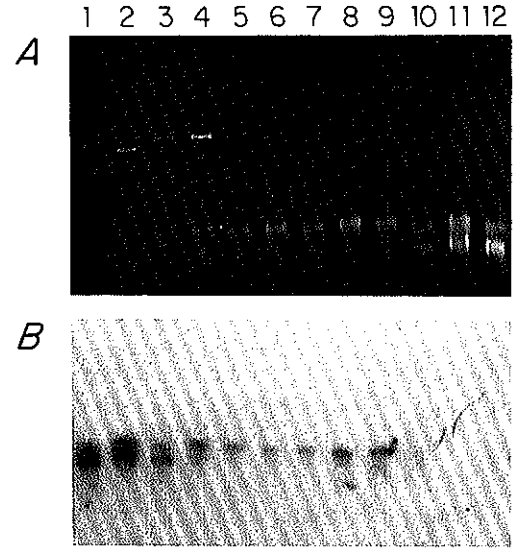

Figure 5.3 Agarose gel electrophoresis of plasmid and chromosomal DNA (A) and Southern hybridization with the ^{32}P-labeled *XbaI-PstI* probe (B). *H. influenzae* strains: lane 1, R1680; lane 2, R1707; lane 3, R1715; and lane 4, R1716. Trimethoprim-resistant transcipients of above strains and R906 are represented in lanes 5, 6, 7 and 8, respectively. Trimethoprim-susceptible transcipients of the above strains in R906 are presented in lanes 9, 10, 11 and 12, respectively.

trimethoprim-susceptible and multiple-resistant transcipients resulting from matings of the wild strains with R906. The probe hybridized in all eight strains with total DNA, but not with plasmid DNA (Figure 5.3). In addition, four Tmp^r transcipients resulting from the mating of R906 with multiple-resistant encapsulated *H. influenzae* were tested. The 0.50-kb probe hybridized only with the total DNA band.

Analysis of DHFR activity in crude enzyme extracts

Measurements of DHFR activity were performed on extracts derived from strains R906, R906/1042-1, and R906/1047-1. All experiments were done in duplicate. Strain R906/1042-1 (inhibited by trimethoprim at 100 μg/ml) had five to six times more DHFR activity per milligram of protein (0.05 and 0.02 U/mg) than the trimethoprim-susceptible recipient, R906 (0.01 and 0.005 U/mg). Extracts from strain R906/1047-1 (MIC, 40 μg/ml) had three to four times greater activity (0.08 and 0.04 U/mg) than extracts from R906. These data support the notion that overproduction of DHFR is the mechanism of trimethoprim resistance in the study strains. The trimethoprim inhibition profiles of the DHFR activity in the three different extracts indicated that 50% inhibition occurred at a trimethoprim concentration of 10^{-8} M with all three cell extracts, suggesting that there was no structural difference in DHFRs of resistant and susceptible strains.

Outer membrane proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane preparations from isogenic strains (R906, R906/1042-1 and R906/1047-1) showed no difference in the number or relative mobility of the proteins in any of the strains. All strains contained 7 major and 13 minor outer membrane proteins.

5.5 DISCUSSION

The incidence of ampicillin-resistant *H. influenzae* in the United States has increased to 20% [14]. Trimethoprim combined with sulfamethoxazole is an effective alternative drug in the treatment of otitis media and sinusitis. However, available data on susceptibility of *H. influenzae* to trimethoprim are inadequate owing to the antagonism of trimethoprim by thymidine in present commercial media [28]. The National Committee for Clinical Laboratory Standards recommends determining trimethoprim susceptibility by agar dilution with Mueller-Hinton agar containing 5% lysed horse blood [39]. Unpublished data from our laboratory show that trimethoprim susceptibility testing on this medium is not reliable: many strains are erroneously identified as resistant. The MICs of trimethoprim for the resistant strains ranged from 10 to < 200 μg/ml. In other species such intermediate levels of resistance appear to be chromosomally encoded [20]. The growth of Tmp^s strains was inhibited by concentrations of < 0.5 μg/ml. The bimodal trimethoprim susceptibility curve of *H. influenzae* strains (susceptible strains for which MICs were < 0.5 μg/ml and resistant strains for which MICs were ≥ 10 μg/ml indicates that trimethoprim resistance in *H. influenzae* is not intrinsic, as in certain *Neisseria* or *Bacteroides* species [57] but is acquired. We could not localize the trimethoprim resistance gene to plasmids in the *H. influenzae* strains, although it was possible to transfer trimethoprim resistance by conjugation. Albritton et al. [1] have previously shown that exchange of chromosomal markers can occur during matings of *H. influenzae* and that the mechanism of transfer is different from classical transformation or conjugation. Gene transfer during conjugation conditions indicates that the trimethoprim resistance gene is linked to the streptomycin and erythromycin resistance genes in *H. influenzae*. Streptomycin and erythromycin both transferred with trimethoprim in conjugation experiments in the absence of selective antibiotic pressure.

We were able to show by cloning the trimethoprim resistance gene and developing a 0.50-kb intragenic probe that considerable homology exists between the gene encoding for trimethoprim resistance and DNA of Tmp^s *H. influenzae*. Southern blot analysis with isogenic Tmp^s and Tmp^r strains showed that the probe hybridized with two *Hin*fI and *Dde*I fragments of different molecular size in the resistant isolate. This indicates that

acquisition of trimethoprim resistance involves some sort of rearrangement or change in nucleotide sequence. Southern blot analysis of separated plasmid and chromosomal DNA derived from multiple-resistant *H. influenzae* confirmed that the trimethoprim resistance gene was chromosomally encoded, in contrast to the plasmid-encoded genes for ampicillin, chloramphenicol, and tetracycline resistance. It is of interest that both chromosome- and plasmid-encoded antibiotic resistance evolved in the same strains. The trimethoprim resistance gene in *H. influenzae* appears to be different from the plasmid-encoded DHFR I, II, and III genes of *E. coli* and from genes encoding for intrinsic trimethoprim resistance of *N. meningitidis* and *N. gonorrhoeae*. Colony hybridization with Tmp^r *P. cepacea* strains also showed no homology with the gene encoding for trimethoprim resistance in these strains. The absence of detectable homology between the trimethoprim resistance gene in *H. influenzae* and genes coding for trimethoprim resistance in other micro-organisms is not surprising, since comparative biochemical and genetic studies of plasmid- and chromosome-encoded DHFRs have shown substantial differences in gene structure, amino acid sequence, and trimethoprim inhibition profiles [8, 18, 54, 62].

Our data suggest that the mechanism of trimethoprim resistance in *H. influenzae* is chromosomally mediated overproduction of DHFR. Outer membrane profiles and trimethoprim uptake studies (not reported) of isogenic Tmp^s and Tmp^r *H. influenzae* strains showed no differences. This indicates that trimethoprim resistance in *H. influenzae* is not caused by decreased penetration of the antibiotic into the cell. Characterization of trimethoprim inhibition profiles in isogenic susceptible and resistant strains further showed a similar 50% inhibition for both susceptible and resistant strains. These data are consistent with the notion that overproduction of DHFR is responsible for trimethoprim resistance but do not exclude the possibility of concomitant synthesis of a trimethoprim-insensitive DHFR. Overproduction of DHFR in other bacterial species and in eukaryotic cells can be caused by gene duplication in the presence of selective trimethoprim pressure [32, 44]. Southern blot analyses do not support this mechanism in *H. influenzae*. Promoter mutation or mutation in a positive or negative regulator gene could lead to increased rates of expression of the DHFR gene in Tmp^r *H. influenzae*. Molecular genetic analysis of the trimethoprim resistance gene in *H. influenzae* will further clarify the genetic basis of resistance in these strains.

Acknowledgments

This work was supported in part by a grant from the Sophia Research Foundation, Rotterdam, The Netherlands.

We are grateful to Fred C. Tenover for kindly reviewing this manuscript.

5.6 REFERENCES

1 **Albritton W.L., Setlow J.K., Slaney L.** *Transfer of Haemophilus influenzae chromosomal genes by cell-to-cell contact.* J Bacteriol 1982;152:1066-1070.

2 **Amyes S.G.B., Smith J.T.** *R-factor trimethoprim resistance mechanism: an insusceptible target site.* Biochem Biophys Res Commun 1974;58:412-418.

3 **Archer G.L., Coughter J.P., Johnston J.L.** *Plasmid-encoded trimethoprim resistance in staphylococci.* Antimicrob Agents Chemother 1986;29:733-740.

4 **Birnboim H.C.** *A rapid alkaline extraction method for the isolation of plasmid DNA.* Methods Enzymol 1983;100:243-255.

5 **Bolivar F., Rodriguez R.L., Greene P.J., Betlach M.C., Heynecker H.L., Boyer H.W., Crosa J.H., Falkow S.** *Construction and characterization of new cloning vehicles. II. A multipurpose cloning system.* Gene 1977;2:95-113.

6 **Boyer H.W., Roulland-Dussoix D.** *A complementation analysis of the restriction and modification of DNA in Escherichia coli.* J Mol Biol 1969;41:459-472.

7 **Braveny I., Machka K.** *Multiply resistant Haemophilus influenzae and parainfluenzae in West Germany.* Lancet 1980;II:752-753.

8 **Burchall J.J.** *Comparative biochemistry of dihydrofolate reductase.* Ann N Y Acad Sci 1971; 186:143-152.

9 **Campos J., García-Tornel S., Gairi J.M., Fábregues I.** *Multiply resistant Haemophilus influenzae type b causing meningitis: comparative clinical and laboratory study.* J Pediatr 1986;108:897-902.

10 **Campos J., García-Tornel S., Sanfellu I.** *Susceptibility studies of multiply resistant Haemophilus influenzae isolated from pediatric patients and contacts.* Antimicrob Agents Chemother 1984;25:706-709.

11 **Catlin B.W.** *Nutritional profiles of Neisseria gonorrhoeae, Neisseria meningitidis and Neisseria lactamica in chemically defined media and the use of growth requirements for gonococcal typing.* J Infect Dis 1973;128:178-194.

12 **Catlin B.W., Bendler III J.W., Goodgal S.H.** *The type b capsulation locus of Haemophilus influenzae: map location and size.* J Gen Microbiol 1972;70:411-422.

13 **De Graaff J., Elwell L.P., Falkow S.** *Molecular nature of two β-lactamase-specifying plasmids isolated from Haemophilus influenzae type b.* J Bacteriol 1976;126:439-446.

14 **Doern G.V., Chapin K.C.** *Susceptibility of Haemophilus influenzae to amoxicillin/clavulanic acid, erythromycin, cefaclor and trimethoprim/sulfamethoxazole.* Diagn Microbiol Infect Dis 1986;4:37-41.

15 **Feigin R.D., Cherry J.D.** *Textbook of pediatric infectious diseases.* The W.B. Saunders Co., Philadelphia 1987.

16 **Flensburg J., Sköld O.** *Massive overproduction of dihydrofolate reductase in bacteria as a response to the use of trimethoprim.* Eur J Biochem 1987;162:473-476.

17 **Fling M.E., Elwell L.P.** *Protein expression in Escherichia coli minicells containing recombinant plasmids specifying trimethoprim-resistant dihydrofolate reductases.* J Bacteriol 1980;141:779-785.

18 **Fling M.E., Richards C.** *The nucleotide sequence of the trimethoprim-resistant dihydrofolate reductase gene harbored by Tn7.* Nucleic Acids Res 1983;11:5147-5158.

19 **Fling M.E., Walton L., Elwell L.P.** *Monitoring of plasmid-encoded, trimethoprim-resistant dihydrofolate reductase genes: detection of a new resistant enzyme.* Antimicrob Agents Chemother 1982;22:882-888.

20 **Grey D., Hamilton-Miller J.M.T., Brumfitt W.** *Incidence and mechanisms of resistance to trimethoprim in clinically isolated Gram-negative bacteria.* Chemotherapy 1979;25:147-156.

21 **Gutmann L., Williamson R., Moreau N., Kitzis M.D., Collatz E., Acar J.F., Goldstein F.W.** *Cross-resistance to nalidixic acid, trimethoprim, and chloramphenicol associated with alterations in outer membrane proteins of Klebsiella, Enterobacter, and Serratia.* J Infect Dis 1985;151:501-507.

22 **Hansen J.B., Olsen R.H.** *Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5.* J Bacteriol 1978;135:227-238.

23 **Herriott R.M., Meyer E.M., Vogt M.** *Defined nongrowth media for stage II development of competence in Haemophilus influenzae.* J Bacteriol 1970;101:517-524.

24 **Hohn B., Collins J.** *A small cosmid for efficient cloning of large DNA fragments.* Gene 1980;11:291-298.

25 **Hull R.A., Gill R.E., Hsu P., Minshew B.H., Falkow S.** *Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection Escherichia coli isolate.* Infect Immun 1981;33:933-938.

26 **Kado C.I., Liu S.T.** *Rapid procedure for detection and isolation of large and small plasmids.* J Bacteriol 1981;145:1365-1373.

27 **Kao J.C., Perry K.L., Kado C.I.** *Indoleacetic acid complementation and its relation to host range specifying genes on the Ti plasmid of Agrobacterium tumefaciens.* Mol Gen Genet 1982;188:425-432.

28 **Koch A.E., Burchall J.J.** *Reversal of the antimicrobial activity of trimethoprim by thymidine in commercially prepared media.* Appl Microbiol 1971;22:812-817.

29 **Lennette E.H., Balows A., Hausler Jr. W.J., Shadomy H.J., (ed.)** *Manual of clinical microbiology 4th ed.* American Society for Microbiology Washington, D.C. 1985.

30 **Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J.** *Protein measurement with the Folin phenol reagent.* J Biol Chem 1951;193:265-275.

31 **Maniatis T., Fritsch E.F., Sambrook J.** *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor N.Y. 1982.

32 **Mariani B.D., Schimke R.T.** *Gene amplification in a single cell cycle in Chinese hamster ovary cells.* J Biol Chem 1984;259:1901-1910.

33 **Maskell R., Okubadejo O.A., Payne R.H., Pead L.** *Human infections with thymine-requiring bacteria.* J Med Microbiol 1977;11:33-45.

34 **McDade Jr. R.L., Johnston K.H.** *Characterization of serologically dominant outer membrane proteins of Neisseria gonorrhoeae.* J Bacteriol 1980;141:1183-1191.

35 **Melton D.A., Krieg P.A., Rebagliati M.R., Maniatis T., Zinn K., Green M.R.** *Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter.* Nucleic Acids Res 1984;12:7035-7056.

36 **Mendelman P.M., Chaffin D.O., Stull T.L., Rubens C.E., Mack K.D., Smith A.L.** *Characterization of non-β-lactamase-mediated ampicillin resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1984;26:235-244.

37 **Meyers J.A., Sanchez D., Elwell L.P., Falkow S.** *Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid.* J Bacteriol 1976;127:1529-1537.

38 **Michalka J., Goodgal S.H.** *Genetic and physical map of the chromosome of Haemophilus influenzae.* J Mol Biol 1969;45:407-421.

39 **National Committee for Clinical Laboratory Standards.** *Methods for agar dilution antimicrobial susceptibility tests for bacteria that grow aerobically.* National Committee for Clinical Laboratory Standards, Villanova, Pa. 1983.

40 **Osborn M.J., Huennekens F.M.** *Enzymatic reduction of dihydrofolic acid.* J Biol Chem 1958;233:969-974.

41 **Pattishall K.H., Acar J., Burchall J.J., Goldstein F.W., Harvey R.J.** *Two distinct types of trimethoprim-resistant dihydrofolate reductase specified by R-plasmids of different compatibility groups.* J Biol Chem 1977;252:2319-2323.

42 **Philpott-Howard J., Williams J.D.** *Increase in antibiotic resistance in Haemophilus influenzae in the United Kingdom since 1977: report of study group.* Br Med J 1982;284:1-8.

43 **Portnoy D.A., Moseley S.L., Falkow S.** *Characterization of plasmids and plasmid-associated determinants of Yersinia enterocolitica pathogenesis.* Infect Immun 1981;31:775-782.

44 **Rigby P.W.J., Burleigh B.D., Hartley B.S.** *Gene duplication in experimental enzyme evolution.* Nature (London) 1974;251:200-204.

45 **Rigby P.W.J., Dieckmann M., Rhodes C., Berg P.** *Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I.* J Mol Biol 1977;113:237-251.

46 **Roberts M.C., Stull T.L., Smith A.L.** *Comparative virulence of Haemophilus influenzae with a type b or type d capsule.* Infect Immun 1981;32:518-524.

47 **Roberts M.C., Swenson C.D., Owens L.M., Smith A.L.** *Characterization of chloramphenicol-resistant Haemophilus influenzae.* Antimicrob Agents Chemother 1980;18:610-615.

48 **Setlow J.K., Boling M.E., Beattie K.L., Kimball R.F.** *A complex of recombination and repair genes in Haemophilus influenzae.* J Mol Biol 1972;68:361-378.

49 **Shurin P.A., Pelton S.I., Donner A., Finkelstein J., Klein J.O.** *Trimethoprim-sulfamethoxazole compared with ampicillin in the treatment of acute otitis media.* J Pediatr 1980;96:1081-1087.

50 **Smith A.L., Smith D.H., Averill Jr. D.R., Marino J., Moxon E.R.** *Production of Haemophilus influenzae b meningitis in infant rats by intraperitoneal inoculation.* Infect Immun 1973;8:278-290.

51 **Smith D.R., Rood J.I., Bird P.I., Sneddon M.K., Calvo J.M., Morrison J.F.** *Amplification and modification of dihydrofolate reductase in Escherichia coli. Nucleotide sequence of fol genes from mutationally altered plasmids.* J Biol Chem 1982;257:9043-9048.

52 **So M., Dallas W.S., Falkow S.** *Characterization of an Escherichia coli plasmid encoding for synthesis of heat-labile toxin: molecular cloning of the toxin determinant.* Infect Immun 1978;21:405-411.

53 **Southern E.M.** *Detection of specific sequences among DNA fragments separated by gel electrophoresis.* J Mol Biol 1975;98:503-517.

54 **Stone D., Smith S.L.** *The amino acid sequence of the trimethoprim-resistant dihydrofolate reductase specified in Escherichia coli by R-plasmid R67.* J Biol Chem 1979;254:10857-10861.

55 **Sundström L., Vinayagamoorthy T., Sköld O.** *Novel type of plasmid-borne resistance to trimethoprim.* Antimicrob Agents Chemother 1987;31:60-66.

56 **Then R.L.** *Mechanisms of resistance to trimethoprim, the sulfonamides, and trimethoprim-sulfamethoxazole.* Rev Infect Dis 1982;4:261-269.

57 **Then R.L., Angehrn P.** *Low trimethoprim susceptibility of anaerobic bacteria due to insensitive dihydrofolate reductases.* Antimicrob Agents Chemother 1979;15:1-6.

58 **Wahl G.M., Stern M., Stark G.R.** *Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate.* Proc Natl Acad Sci USA 1979;76:3683-3687.

59 **Werner R.G., Goeth H.** *Trimethoprim, failure to penetrate into Pseudomonas aeruginosa cells.* FEMS Microbiol Lett 1984;23:201-204.

60 **Wray W., Boulikas T., Wray V.P., Hancock R.** *Silver staining of proteins in polyacrylamide gels.* Anal Biochem 1981;118:197-203.

61 **Zackrisson G., Brorson J.-E.** *Antibiotic sensitivity of Haemophilus influenzae strains including three recent chloramphenicol-resistant isolates.* Acta Pathol Microbiol Scand Sect B 1980;88:193-198.

62 **Zolg J.W., Hanggi U.J.** *Characterization of R-plasmid-associated, trimethoprim-resistant dihydrofolate reductase and determination of the nucleotide sequence of the reductase gene.* Nucleic Acids Res 1981;9:697-710.

ANTIBIOTIC RESISTANCE IN HAEMOPHILUS INFLUENZAE

CHAPTER 6

TRIMETHOPRIM RESISTANCE IN HAEMOPHILUS INFLUENZAE IS DUE TO ALTERED DIHYDROFOLATE REDUCTASE(S)

Biochem J 1991;274:657-662.

CHAPTER 6

TRIMETHOPRIM RESISTANCE IN HAEMOPHILUS INFLUENZAE IS DUE TO ALTERED DIHYDROFOLATE REDUCTASE(S)

Ronald de Groot[1,2], Donald O. Chaffin[1], Meta Kuehn[1] and Arnold L. Smith[1,2].

[1] *Division of Infectious Disease, Children's Hospital and Medical Center, Seattle, USA.*

[2] *Department of Pediatrics, University of Washington, Seattle, USA.*

6.1 SUMMARY

We characterized a highly purified preparation of the chromosomally encoded dihydrofolate reductase (DHFR) from a trimethoprim-susceptible (Tmp^s; strain MAP) and two trimethoprim-resistant (Tmp^r) strains (MAP/47 and MAP/42) of *Haemophilus influenzae*. The enzymes were purified between 650- and 3000-fold by gel-filtration and dye-ligand chromatography. The apparent molecular mass of the three proteins was 18400 Da by PAGE under denaturing and non-denaturing conditions. Total enzyme activity was greater in all fractions from the Tmp^r strains compared with the Tmp^s isolate. The three enzymes had a similar K_m for dihydrofolate (7, 9 and 5 μM) and NADPH (2, 5 and 6 μM). However, the Tmp IC_{50} (the concentration necessary for 50% inhibition of DHFR activity) for the Tmp^s strain MAP was 0.001 μM, whereas DHFR from the Tmp^r strains MAP/47 and MAP/42 had values of 0.1 μM and 0.3 μM respectively. The methotrexate IC_{50} of the MAP/42 DHFR was 0.06 μM in comparison with the enzyme from MAP (0.008 μM) and MAP/47 (0.007μM). Isoelectric focusing indicated that the DHFR from MAP/42 had a different isoelectric point (pI 7.6) compared with the enzymes from MAP and MAP/47 (pI 7.3). Peptide mapping after digestion with trypsin revealed one major peptide fragment (7.9 kDa) in the DHFR of MAP and MAP/47 and three major tryptic fragments (7.9, 9.6 and 12.5 kDa) in DHFR from MAP/42. We conclude that trimethoprim resistance in *H. influenzae* results from overproduction of structurally altered DHFR(s).

6.2 INTRODUCTION

Bacterial resistance to trimethoprim (Tmp) is commonly due to resistance plasmids which encode (a) dihydrofolate reductase(s) [DHFR(s)] with lower affinity for Tmp. There are six classes of R-plasmid-encoded DHFRs in Gram-negative micro-organisms [11, 26, 30, 33, 37, 38] and one class in Gram-positive bacteria [1]. Additional mechanisms of resistance include chromosomal mutation leading to overproduction of DHFR [10, 32], a decreased outer-membrane permeability to Tmp [14, 35] or thymine auxotrophy [21].
DHFRs from several bacteria, e.g., *Neisseria gonorrhoeae*, *Streptococcus faecium*, *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pneumoniae*, have been partially purified and characterized [2, 23, 24, 29, 38]. The amino acid sequence of these enzymes shows considerable inter- and intra-species differences [2, 12, 31], even though they may possess common kinetic properties.
We previously reported, that Tmp resistance in *Haemophilus influenzae* was chromosomally mediated and associated with increased DHFR activity in sonicated cells [13]. We describe here the partial purification and properties of DHFR from three strains, two of which are trimethoprim-resistant. All are isogenic, except for the Tmp^r determinant. All three enzymes have a molecular mass of approx. 18400 Da, but have structural and functional differences.

6.3 MATERIALS AND METHODS

Bacterial strains

Strain MAP [6] (previously described as strain R906 [13]) is Tmp^s (minimal inhibitory concentration (MIC) 0.5 μg/ml). It was used as a recipient for transformation with DNA derived from Tmp^r strains R1047 (MIC 30 μg/ml) and R1042 (MIC 100 μg/ml) [13]. In the present study, two trimethoprim- and streptomycin-resistant transformants shown to be erythromycin-resistant were identified as MAP/1047-1 and MAP/1042-1 respectively [13]. These previously described isogenic *Haemophilus influenzae* strains were used in the present study: a Tmp^s strain [MAP (MIC 0.5 μg/ml)], a low-level Tmp^r strain [MAP/1047-1 (MIC 30 μg/ml), called MAP/47 here] and a high-level Tmp^r strain [MAP/1042-1 (MIC 100 μg/ml, called MAP/42 here)].

Media

Haemophilus influenzae were grown in brain/heart-infusion agar or broth (Difco) supplemented with 10 μg of haemin chloride/ml, 10 μg of histidine/ml, and 2 μg of β-NAD^+/ml

(sBHI) [13]. Solid media were incubated overnight at 36.5°C in 5% CO_2, whereas liquid cultures were incubated at 37°C in air and shaken at 150 rev./min.

Materials

Dihydrofolic acid (DHF), β-NADPH, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethane sulphonyl fluoride, dithiothreitol, Tmp and methotrexate were obtained from Sigma Chemical Co. (St Louis, Mo, U.S.A.). The antibiotic stock solution of Tmp used for determination of the 50%-inhibitory constant (IC_{50}) was 5 mg/ml (17.24 mM) in 50 mM-HCl. The stock solution of methotrexate was 4.55 mg/10 ml of distilled water (1mM). They were stored at 4°C. Sephadex G-75 was supplied by Pharmacia, Uppsala, Sweden. Matrix Gel Green A, ultrafiltration cell and YM 10 membranes were purchased from Amicon Corporation (Lexington, Ma, U.S.A.). Ampholines (pH 5-8) were obtained from LKB (Bromma, Sweden). Sequencing-grade trypsin was obtained from Boehringer-Mannheim (Indianapolis, In, U.S.A.). Protein molecular-mass standards, ultrapure glycerol and $(NH_4)_2SO_4$ were supplied by BRL (Gaithersburg, Md, U.S.A.). Bradford protein assay mixture was purchased from Bio-Rad (Richmond, Ca, U.S.A.). All other chemicals were of reagent grade.

Enzyme purification

Cultures (4 litres) of each strain were grown aerobically overnight to about 10^{10} cells/ml in sBHI. Cells were pelleted at 10000 g and frozen at -20°C until the subsequent steps. All subsequent steps were performed at 4°C. Cell pellets from a total of 12 litres of culture (approx. 12g of cells) were resuspended in 120 ml of 50 mM-sodium phosphate buffer, pH 7.4, and pelleted at 10000 g. The pellets were resuspended in 120 ml of phosphate buffer, pH 7.4, containing 1 mM-dithiothreitol, 1 mM-sodium EDTA, 50 mM-KCl and 0.1 mM-phenylmethane sulphonyl fluoride and sonicated at 20 W at 0-4°C for 30 s ten times (5 min total) in a W370 cell disruptor (Heat Systems Ultrasonics). After centrifugation of the sonicated cells at 10000 g for 1 h, the supernatant was collected and DNAase was added to yield a final concentration of 50 μg/ml. The solution was incubated at room temperature for 15 min and centrifuged at 10000 g for 30 min. Remaining nucleic acids were precipitated by the addition of 0.1 vol. of 5% (w/v) streptomycin sulphate solution in 50 mM phosphate buffer, pH 7.4.

After centrifugation the supernatant was used for $(NH_4)_2SO_4$ fractionation. The fraction precipitating between 45 to 80% saturation contained more than 90% of the DHFR activity. The $(NH_4)_2SO_4$ pellet was dissolved in 20 ml of 50 mM-phosphate buffer, pH 7.4, dialysed overnight against the same buffer and applied to a Sephadex G-75 column (75 cm x 1.25 cm). The sample was eluted with the same buffer containing 20% (v/v) glycerol

fractions were assayed for DHFR activity. Those with DHFR activity were pooled and applied to a Matrix Gel Green A column (2.6 cm x 40 cm) equilibrated with 50 mM-phosphate buffer. The column was washed for a minimum of 24 h with 540 ml of the same buffer: this effluent did not contain detectable DHFR activity. The samples were then eluted at 10 ml/h with the same buffer containing 20% (v/v) ultrapure glycerol and a linear salt gradient (0-2 M-KCl). Fractions with DHFR activity were pooled and concentrated at 345 kPa (50 lbf/in^2) in an Amicon ultrafiltration cell with a YM-10 membrane and stored at 4°C.

Enzyme assay

DHFR activity was measured spectrophotometrically with a Beckman model DU 6 spectrophotometer as described previously [25]. Enzyme activity was detected by measuring the DHF-dependent decrease in the absorbance at 340 nm with time at 30°C. The standard enzyme assay contained 0.1 mmol of NADPH, 50 mM-sodium phosphate buffer, pH 7.4 and 10 μM-mercaptoethanol with or without DHF (0.1 mmol) in a final volume of 1 ml. The linear decrease in absorbance was measured during the first 30 to 60 s to estimate initial velocity. The decrease in absorbance was corrected for that occurring in the absence of DHF and enzyme source. One enzyme unit was defined as the amount required to reduce 1 μmol of DHF/min, [on the basis of a molar absorption coefficient of 12.3 x 10^3 litre.mol^{-1}.cm^{-1}] [17]. Specific activity was expressed by enzyme units/min per mg of protein. Studies of enzyme kinetics were performed with DHFR eluted from the Green A matrix gel. Kinetic parameters at different concentrations of substrate, cofactor or inhibitors were obtained by three to five replicate measurements. The K_m for DHF and NADPH was estimated from the equation:

$$V_i = (V_{max} \cdot [S]) / (K_m + [S])$$

in which V_i and [S], V_{max} and K_m are (respectively) initial velocity, substrate concentration, maximum reaction velocity and Michaelis-Menten constant. The means ± S.D. for the replicates and the P values were calculated by using the methods of Bevington and Choi [3, 7]. The Michaelis-Menten kinetic analyses were performed by preincubating DHF and NADPH for 3 min before starting the reaction with enzyme source. IC_{50} was determined for all three preparations using different concentrations of Tmp or methotrexate with DHF and NADPH concentration constant at 0.1 mM.

Tmp and methotrexate IC_{50} values were calculated by using the equation:

$$v = V_{max} - (V_{max} \cdot [I]) / (IC_{50} + [I])$$

in which v, V_{max} and [I] are (respectively) observed velocity, maximum reaction velocity

and concentration of the inhibitor. IC_{50} values were obtained after preincubation of NADPH, enzyme source and inhibitor for 3 min. The reaction was then started by adding DHF. A non-linear least-squares fit was used to determine the values of V_{max} and IC_{50}.

Protein determination

Protein concentration of samples was measured as described by Bradford [4], with BSA as the standard.

PAGE

SDS/PAGE was performed on 10-12%-acrylamide separating gels with 4%-acrylamide stacking gels, using the method of Laemmli [20]. Electrophoresis was continued at 20°C and 50 V until the Bromophenol Blue marker migrated to the bottom of the gel. Proteins were detected by using the silver-staining technique of Wray et al. [36]. Non-denaturing continuous gel electrophoresis was performed on 10%-acrylamide slab gels as described by Hames [15]. Enzyme preparations were suspended in 10% (w/v) sucrose/0.2% Bromophenol Blue/ 10mM Tris/HCl, pH 8.0. Samples were electrophoresed for 16 h at 140 V. DHFR activity in the gel was detected by using the system described by Hiebert et al. [16]. NADPH (8.3 mg), DHF (2.2 mg), MTT (16.5 mg) and 0.5 M-Tris/HCl, pH 7.4 (5 ml) were mixed in a total volume of 50 ml. The gels were incubated in this solution for 15 min at 37°C, at which time blue bands were evident. These bands were cut from the gel and minced into small pieces. The minced fragments were resuspended in running buffer, subjected to SDS/12%-PAGE and silver-stained as described above to estimate molecular mass.

Isoelectric focusing

Isoelectric focusing was performed under non-denaturing conditions in a tube-gel electrophoresis system (Bio-Rad model 150A; Bio-Rad, Richmond, Ca, U.S.A.). Gels were prepared as described by Eder [9]. The lower reservoir was filled with 0.05 M-H_2SO_4 as anode solution and the upper chamber was filled with 0.03 M-NaOH as the cathode solution. The gels were prerun at 1 mA/gel for 15 min. Enzyme preparations in 50 mM-phosfate buffer, pH 7.4, containing 10% sucrose, and 6% Ampholines (pH 5-8) were loaded, focused for 7 h and DHFR activity detected by the method of Hiebert et al. [16] described above. The pH gradient was measured by slicing an unstained gel into 0.5 cm sections, suspending each section in 1 ml of deionized water, equilibrating at 21°C for 1 h and measuring the pH. Bands with DHFR activity were excised from the gel, minced into small pieces, and subjected to SDS/12%-PAGE; proteins were detected by silver stain as described above.

Peptide mapping

Peptide mapping was performed as described by Cleveland et al. [8]. After Green A matrix gel chromatography, fractions with DHFR activity derived from strains MAP, MAP/47 and MAP/42 were electrophoresed on SDS/15%-PAGE minigels. The predominant 18.4 kDa band was excised from the gel after revealing protein bands with Ponceau S stain [27]. The gel fragments were placed in 0.125 M-Tris/HCl (pH 8.0)/0.1% SDS/1mM-EDTA and electroeluted in a Bio-Rad model-422 electroeluter using 50 mM-NH_4HCO_3 (pH 8.0)/0.1% SDS. Elution was performed at 10 mA/sample for 4 h. Each sample was freeze-dried in a Speed-Vac (Savant Instruments), and 100 mg of trypsin in 1 ml of 1 mM-HCl was added to each sample. A gel fragment not containing detectable protein was processed as a control. Digestion was continued for 18 h at 38°C. Samples were then dried and stored at 4°C. The tryptic digests were resuspended in 50 μl of 0.1M-sodium phosphate, pH 7.4, and radiolodinated with $Na^{125}I$ by the chloramine-T procedure [22]. ^{125}I-labelled samples and molecular-mass standards were electrophoresed overnight on a SDS/15%-PAGE gel. The gel was dried after fixing in methanol/acetic acid/water (9:2:9, by vol.) for 1 hour. Autoradiographs were made after 20 min, 30 min, 1 h, 4.5 h and 18 h of exposure, with a screen between gel and film to intensify the bands.

6.4 RESULTS

Enzyme purification

Initial attempts at purification of DHFR were associated with loss of enzyme activity in the steps after $(NH_4)_2SO_4$ fractionation. This problem was solved by the addition of 20% (v/v) glycerol during gel-filtration steps. The purification scheme for DHFR from the three strains is summarized in Table 6.1. These data are the average of three replicate purifications using frozen cells from a total of 12 1-litre cultures of each strain. Total enzyme activity was calculated after correction for DHF-independent NADPH oxidase activity. This activity was considerable in the first two steps of the purification procedure, but present in minute quantities after $(NH_4)_2SO_4$ precipitation, and not detectable after the gel-filtration steps. Total enzyme activity of the final electrophoretically pure preparations from MAP/47 and MAP/42 was 6-10-fold higher than those derived from MAP. The specific activity of DHFR derived from these strains was 8-fold the specific activity of DHFR from MAP in the initial fractions, but became similar as the enzyme was purified. Purification for the three enzymes varied between 650- to more than 3000-fold, with recovery of total activity between 16 and 29%.

Table 6.1 Purification of DHFR from isogenic *H. influenzae* strains.

Strain	Fraction[a]	Total enzyme activity (units[b])	Total Protein (mg)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
MAP	1	6016	2693	2.2	1	100
	2	5691	2537	2.2	1	95
	3	5285	1441	3.7	1.3	88
	4	2195	9	243.9	103	36
	5	1220	0.2	6097.5	3193	20
MAP/47	1	49024	3174	15.5	1	100
	2	49024	3050	16.1	1	100
	3	48862	834	58.6	3.8	100
	4	43252	23	1880.5	120	88
	5	13984	0.8	17479.6	1132	29
MAP/42	1	47642	2856	16.7	1	100
	2	57480	2641	21.8	1.3	121
	3	56179	1045	53.8	3.1	118
	4	18211	40	455.3	27	38
	5	7724	0.9	8581.8	648	16

[a] Fractions are identified as follows: 1, cell lysate; 2, after streptomycin sulfate treatment; 3, after $(NH_4)_2SO_4$ fractionation; 4, after Sephadex G-75; 5, after Green A Matrix chromatography.

[b] μmol of tetrahydrofolate produced/min from dihydrofolate and NADPH [17].

Molecular mass

The molecular masses of the native enzymes were estimated by gel-filtration on Sephadex G-75 in comparison with reference proteins. DHFR activity was eluted as a single activity peak corresponding to a molecular mass of approximately 18000 Da. SDS/PAGE of fractions with high specific activity indicated a protein with a relative mobility equivalent to 18.4 kDa (Figure 6.1). When the same enzyme fractions were analyzed by non-denaturing gel electrophoresis, we detected DHFR activity in the lanes containing extract of MAP and MAP/47, but not from MAP/42. Bands with DHFR activity were electroeluted from the gel and analyzed by SDS/PAGE; each contained a single protein with a mobility equivalent to 18.4 kDa.

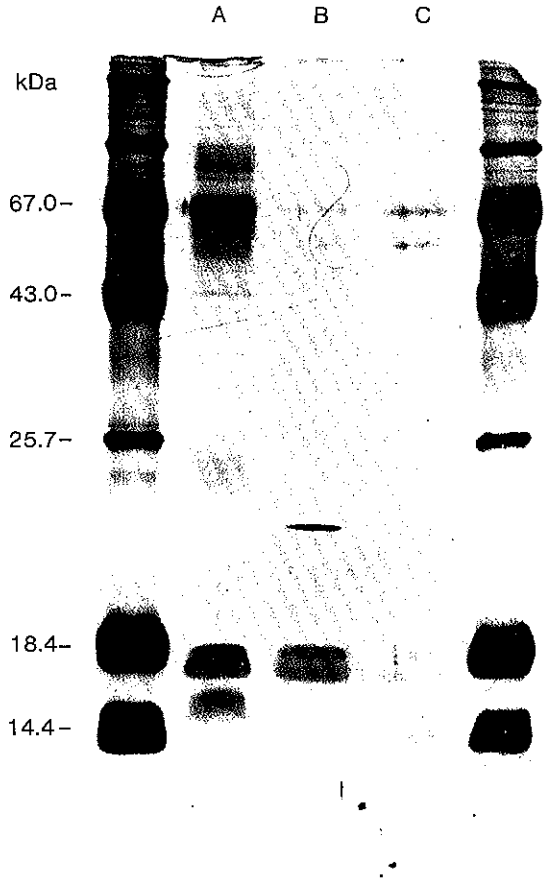

Figure 6.1 SDS/PAGE analysis of purified MAP, MAP/47 and MAP/42 DHFR.

Molecular-size standards are shown in the extreme left and right lanes. Lane A, 2,8 μg of protein from MAP/42; B, 2.8 μg of protein from MAP/47; and lane C, 2.8 μg of protein of partially purified DHFR from strain MAP.

Isoelectric focusing

Fraction 5 derived from the strains was subjected to isoelectric focusing and DHFR activity was detected in the gel as described above. Fraction 5 from MAP and MAP/47 had a different pI compared with MAP/42 (Figure 6.2). A major band corresponding with a pI of 7.6 was seen with MAP/42, whereas a pI of 7.3 was found with the fractions from MAP and MAP/47. When fraction 3 of each strain was subjected to isoelectric focusing, a faint band with a pI of 7.1 could be seen in fractions from MAP and MAP/47. The apparent molecular mass of the fraction containing most of the DHFR activity was confirmed by elution from the isoelectric-focusing gel followed by SDS/PAGE; the protein had a relative mobility equivalent to 18.4 kDa.

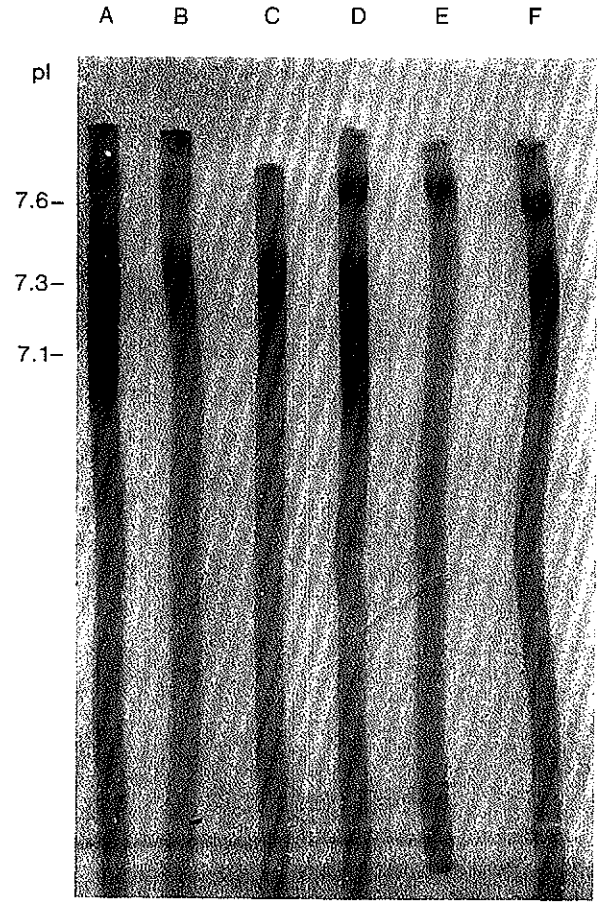

Figure 6.2 Isoelectric focusing of single fractions and combinations of DHFR from strains MAP, MAP/47 and MAP/42.

DHFR was detected as described in the text. MAP samples contained 1.75 units of DHFR and 8.75 μg of protein; the MAP/47 sample contained 11.2 units of DHFR and 2.15 μg of protein; and the MAP/42 sample contained 11.0 units of DHFR and 12 μg of protein. Lane A, MAP and MAP/47; B, MAP; C, MAP/47; D, MAP/47 and MAP/42; E, MAP/42; F, MAP and MAP/42.

Enzyme kinetics

The kinetics of fraction 5 of each DHFR were studied to determine whether functional differences existed. Kinetic parameters including K_m for DHF, K_m for NADPH and IC_{50} for Tmp and methotrexate are summarized in Table 6.2 and depicted in Figure 6.3. Statistical analysis using the Aspen-Welch test [7] showed significant differences in K_m for DHF between MAP and MAP/42 (< 0.05), whereas those between MAP and MAP/47 were not significantly different ($p > 0.1$). In addition, significant differences in K_m for NADPH were found between MAP and MAP/47 ($p < 0.05$) and MAP and MAP/42 ($p < 0.025$). However, K_m values for DHF and NADPH showed less than a 3-fold difference between the three strains. In contrast, approx. 100- and 200-fold differences were measured between the Tmp IC_{50} values of MAP and MAP/47 ($p < 0.0005$) and MAP and MAP/42

Table 6.2 Comparison of kinetic properties[a] of various *H. influenzae* DHFRs.

Strain	K_m DHF (μM)	K_m NADPH (μM)	Trimethoprim IC_{50} (μM)	Methotrexate IC_{50} (μM)
MAP	6.71 ± 1.14	2.01 ± 0.19	0.0013 ± 0.0002	0.0066 ± 0.0018
MAP/47	8.60 ± 0.64	5.05 ± 0.85	0.14 ± 0.005	0.0083 ± 0.001
MAP/42	4.75 ± 0.81	6.00 ± 0.68	0.26 ± 0.03	0.062 ± 0.001

[a] Results are means ± S.D. for three or four replicates.

($p < 0.005$) respectively. In addition, a 9-fold difference was found between the methotrexate IC_{50} values of MAP and MAP/42 ($p < 0.0005$), whereas no significant difference was observed between the methotrexate IC_{50} values of MAP and MAP/47.

Peptide mapping

Proteolytic digestion of fraction 5 from the three strains resulted in the generation of one major peptide of 7.9 kDa in DHFR from strains MAP and MAP/47 and three major peptides (7.9, 9.6 and 12.5 kDa) from strain MAP/42. In addition, an 18.4 kDa band and a 22 kDa trypsin band were seen on the autoradiographs.

6.5 DISCUSSION

Assaying crude cell lysates, we suggested that the mechanism of Tmp resistance in *H. influenzae* was overproduction of chromosomally encoded DHFR [13]. In the present study, DHFRs from an isogenic Tmp^s and two Tmp^r *H. influenzae* strains were highly purified. The purification scheme involving gel-filtration and dye-ligand chromatography was similar to that used for DHFR from *E. coli* R483 [24]. A high degree of purification (650-3000-fold) was obtained, with total recovery of enzyme activity varying between 16 and 29%. The relative instability of DHFR was eliminated by adding 20% glycerol to the phosphate buffer. Total DHFR activity recovered from the strains was 6-10-fold higher in preparations from Tmp^r strains compared with the Tmp^s strain. Chromosomal mutations resulting in overproduction of DHFR have previously been described in *Streptococcus pneumoniae*, *Neisseria gonorrhoeae* and *Escherichia coli* [2, 28, 29].

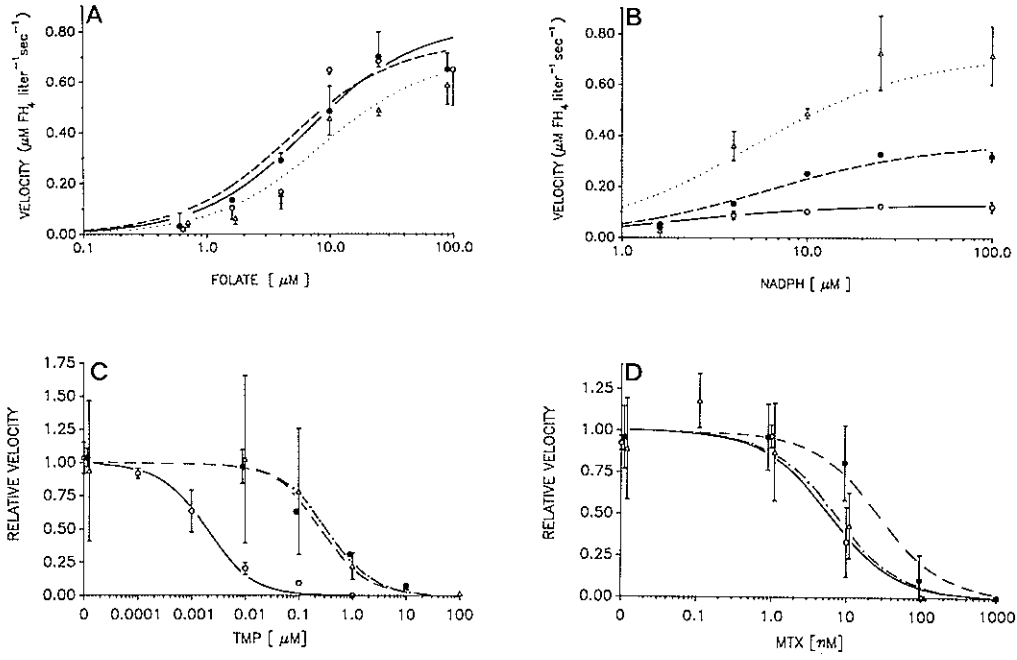

Figure 6.3 Kinetic parameters of DHFR from MAP, MAP/47 and MAP/42.

(A, B) Initial velocity in the presence of different concentrations of DHF (A) and NADPH (B). For details, see the Materials and methods section. Strains: o—o, MAP; Δ—Δ, MAP/47; •—•, MAP/42. (C, D) Inhibition of DHFR from MAP (o—o), MAP/47 (Δ—Δ) and MAP/42 (•—•) by Tmp (C) and methotrexate (D). For details, see the Materials and methods section.

The K_m and V_{max} for DHF and NADPH of the three DHFRs varied by 2-3-fold, a difference we considered to be physiologically unimportant. In contrast, the Tmp IC_{50} for the enzymes from MAP/47 and MAP/42 Tmp^r strains was 100- and 200-fold higher than the Tmp IC_{50} for the DHFR from Tmp^s MAP. The methotrexate IC_{50} for MAP/42 was approx. 10-fold higher than the IC_{50} values for MAP and MAP/47. These data suggest that Tmp resistance in *H. influenzae* is caused not only by overproduction of DHFR, but also by changes in enzyme structure, resulting in a lower affinity of DHFR for antifolates. Comparison of the kinetic parameters from a variety of chromosomally and plasmid-encoded DHFRs (Table 6.3) shows that the values of *H. influenzae* DHFR K_m for DHF and NADPH are similar to those found with chromosomally and plasmid-mediated DHFR in *E. coli* and *N. gonorrhoeae*. Similarly, the trimethoprim IC_{50} of Tmp^s *H. influenzae* is in the same range as Tmp^s *E. coli*. The trimethoprim IC_{50} values for DHFR derived from Tmp^r *H. influenzae* are intermediate between those from Tmp^s *E. coli* DHFR and that of the plasmid-encoded DHFR from Tmp^r *E. coli*. This is not surprising in view of the relatively

Table 6.3 Michaelis-Menten and inhibition constants of DHFRs in various micro-organisms[a].

Micro-organism	Strain	Source	MIC (μg/ml)	DHF K_m (μM)	NADPH K_m (μM)	Tmp IC_{50} (μM)	MTX IC_{50} (μM)	Reference
H. influenzae	MAP	C	0.5	6.7	2.0	0.001	0.007	[13]
	MAP/47	C	30	8.6	5.1	0.1	0.008	[13]
	MAP/42	C	100	4.8	6.1	0.3	0.06	[13]
E. coli	J5	C	0.2	1.2	7.0	0.02-0.007	0.004	[26]
	R483	P	> 1000	5.6	12.3-17.3	3.8-57	0.3-4.4	[24, 26, 34]
	R67	P	> 2000	4.1	6.4	70,000	1000	[26, 34]
	pAZ1	P	64	0.4	N.D.	2.1	N.D.	[19]
	pUK1123	P	160	37	N.D.	0.2	0.02	[38]
N. gonorrhoeae	F62	C	48	2.1	13	N.D.	0.0002	[2]
	T47	C	1200	2.6	10	N.D.	0.0002	[2]

[a] Abbreviations: MIC, minimal inhibitory concentration; DHF, dihydrofolic acid; Tmp, trimethoprim; MTX, methotrexate; ND, not determined; C, chromosome; P, plasmid.

low Tmp MICs of Tmp^r *H. influenzae* strains (30 μg/ml and 100 μg/ml) compared with the higher MICs (> 1000 μg/ml) of certain plasmid-encoded enzymes from Tmp^r *E. coli*.

The apparent molecular mass of all these *H. influenz*ae enzymes was 18000 Da. This is similar to the molecular mass of chromosomally and plasmid-mediated DHFR derived from other Gram-positive and Gram-negative micro-organisms [2, 19, 26, 34]. Isoelectric focusing confirmed that the pI of MAP/42 DHFR was different (7.6) from those of MAP and MAP/47 DHFRs (7.3). These values are substantially higher than the pI of DHFR from chromosomal *E. coli* (4.4), but in the same range as the pI of certain plasmid-encoded DHFRs from Tmp^r *E. coli* [5, 18, 26]. When crude fractions from MAP and MAP/47 were allowed to react longer, or more protein was applied to the gel, an additional band corresponding to a pIs of 7.1 could be seen (Figure 6.2). We speculate that this second band might be an artefact, as was shown by Baccanari et al. (1984) with DHFR from *N. gonorrhoeae* [2]. Alternatively the endogenous DHFR might be detected. The presence of structural differences between DHFRs from MAP/42 and the other strains was supported by the peptide maps. Only one major tryptic peptide fragment was seen in DHFRs from MAP and MAP/47, whereas three major fragments were observed in DHFR from MAP/42.

Using DHFR preparations purified 600-3000-fold, we found structural and functional differences between the DHFR of the three strains. We suggest that the genetic basis for these changes may be similar to those encountered in regulatory mutants of DHFR in *E. coli* K12 [28]. Studies by Smith & Calvo [31] revealed that the genetic basis of Tmp^r induced by mutagenesis was a mutation in the "fol" gene, resulting in a DHFR with lower affinity for Tmp, and a second mutation in the promoter, resulting in an increased synthesis of DHFR-specific mRNA.

6.6 REFERENCES

1 **Archer G.L., Coughter J.P., Johnston J.L.** *Plasmid-encoded trimethoprim resistance in Staphylococci.* Antimicrob Agents Chemother 1986;29:733-740.

2 **Baccanari D.P., Tansik R.L., Paterson S.J., Stone D.** *Characterization and amino acid sequence of Neisseria gonorrhoeae dihydrofolate reductase.* J Biol Chem 1984;259:12291-12298.

3 **Bevington P.R.** *Data reduction and error analysis for the physical sciences.* McGraw-Hill, New York 1969;72-74.

4 **Bradford M.M.** *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem 1976;72:248-254.

5 **Broad D.F., Smith J.T.** *Classification of trimethoprim-resistant dihydrofolate reductases mediated by R-plasmids using isoelectric focusing.* Eur J Biochem 1982;125:617-622.

6 **Catlin B.W., Bendler III J.W., Goodgal S.H.** *The type b capsulation locus of Haemophilus influenzae: map location and size.* J Gen Microbiol 1972;70:411-422.

7 **Choi A.J.,** *Introductory applied statistics in science.* Prentice-Hall, Englewood Cliffs, N J 1978;136-137.

8 **Cleveland D.W., Fischer S.G., Kirschner M.W., Laemmli U.K.** *Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis.* J Biol Chem 1977;252:1102-1106.

9 **Eder J.** *Isoelectric focusing of antibodies in polyacrylamide gels.* J Immunol Meth 1972;2:67-74.

10 **Flensburg J., Sköld O.** *Massive overproduction of dihydrofolate reductase in bacteria as a response to the use of trimethoprim.* Eur J Biochem 1987;162:473-476.

11 **Fling M.E., Walton L., Elwell L.P.** *Monitoring of plasmid-encoded, trimethoprim-resistant dihydrofolate reductase genes: detection of a new resistant enzyme.* Antimicrob Agents Chemother 1982;22:882-888.

12 **Fling M.E., Richards C.** *The nucleotide sequence of the trimethoprim-resistant dihydrofolate reductase gene harbored by Tn7.* Nucleic Acids Res 1983;11:5147-5158.

13 **de Groot R., Campos J., Moseley S.L., Smith A.L.** *Molecular cloning and mechanism of trimethoprim resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1988;32:477-484.

14 **Gutmann L., Williamson R., Moreau N., Kitzis M.D., Collatz E., Acar J.F., Goldstein F.W.** *Cross-resistance to nalidixic acid, trimethoprim and chloramphenicol associated with alterations in outer membrane proteins of Klebsiella, Enterobacter, and Serratia.* J Infect Dis 1985;151:501-507.

15 **Hames B.D.** *Gel-electrophoresis of proteins: a practical approach.* (Hames B.D., Richwood D., eds.). IRL Press, London, 1981.

16 **Hiebert M., Gauldie J., Hillcoat B.L.** *Multiple enzyme forms from protein-bromphenol blue interaction during gel electrophoresis.* Anal Biochem 1972;46:433-437.

17 **Hillcoat B.L., Nixon P.F., Blakley R.L.** *Effect of substrate decomposition on the spectrophotometric assay of dihydrofolate reductase.* Anal Biochem 1967;21:178-189.

18 **Huovinen P.** *Trimethoprim resistance.* Antimicrob Agents Chemother 1987;31:1451-1456.

19 **Joyner S.S., Fling M.E., Stone D., Baccanari D.P.** *Characterization of an R-plasmid dihydrofolate reductase with a monomeric structure.* J Biol Chem 1984;259:5851-5856.

20 **Laemmli U.K.** *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature (London) 1970;227:680-685.

21 **Maskell R., Okubadejo O.A., Payne R.H., Pead L.** *Human infections with thymine-requiring bacteria.* J Med Microbiol 1977;11:33-45.

22 **McConahey P.J., Dixon F.J.** *Radioiodination of proteins by the use of the chloramine-T method.* Methods Enzymol 1980;70:210-213.

23 **Nixon P.F., Blakley R.L.** *Dihydrofolate reductase of Streptococcus faecium.* J Biol Chem 1968;243:4722-4731.

24 **Novak P., Stone D., Burchall J.J.** *R-plasmid dihydrofolate reductase with a dimeric subunit structure.* J Biol Chem 1983;258:10956-10959.

25 **Osborn M.J., Huennekens F.M.** *Enzymatic reduction of dihydrofolic acid.* J Biol Chem 1958;233:969-974.

26 **Pattishall K.H., Acar J.F., Burchall J.J., Goldstein F.W., Harvey R.J.** *Two distinct types of trimethoprim-resistant dihydrofolate reductase specified by R-plasmids of different compatibility groups.* J Biol Chem 1976;252:2319-2323.

27 **Salinovich O., Montelaro R.C.** *Reversible staining and peptide mapping of proteins transferred to nitrocellulose after separation by sodium dodecylsulfate-polyacrylamide gel electrophoresis.* Anal Biochem 1986;156:341-347.

28 **Sheldon R.** *Altered dihydrofolate reductase in fol regulatory mutants of Escherichia coli K12.* Mol Gen Genet 1977;151:215-219.

29 **Sirotnak F.M., Hachtel S.L., Williams W.A.** *Increased dihydrofolate reductase synthesis in Diplococcus pneumoniae following translatable alteration of the structural gene. II. Individual and dual effects of the properties and rate of synthesis of the enzyme.* Genetics 1969;61:313-326.

30 **Sköld O., Widh A.** *A new dihydrofolate reductase with low trimethoprim sensitivity induced by an R-factor mediating high resistance to trimethoprim.* J Biol Chem 1974;249:4324-4325.

31 **Smith D.R., Calvo J.M.** *Nucleotide sequence of the E. coli gene coding for dihydrofolate reductase.* Nucleic Acids Res 1980;8:2255-2274.

32 **Smith D.R., Calvo J.M.** *Nucleotide sequence of dihydrofolate reductase genes from trimethoprim-resistant mutants of Escherichia coli.* Mol Gen Genet 1982;187:72-78.

33 **Sundström L., Vinayagamoorthy T., Sköld O.** *Novel type of plasmid-borne resistance to trimethoprim.* Antimicrob Agents Chemother 1987;31:60-66.

34 **Tennhammar-Ekman B., Sköld O.** *Trimethoprim resistance plasmids of different origin encode different drug-resistant dihydrofolate reductases.* Plasmid 1979;2:334-346.

35 **Werner R.G., Goeth H.** *Trimethoprim, failure to penetrate into Pseudomonas aeruginosa cells.* FEMS Microbiol Lett 1984;23:201-204.

36 **Wray W., Boulikas T., Wray V.P., Hancock R.** *Silver-staining of proteins in polyacrylamide gels.* Anal Biochem 1981;118:197-203.

37 **Wylie B.A., Amyes S.G.B., Young H.K., Koornhof H.J.** *Identification of a novel plasmid-encoded dihydrofolate reductase mediating high-level resistance to trimethoprim.* J Antimicrob Chemother 1988;22:429-435.

38 **Young H.K., Amyes S.G.B.** *A new mechanism of plasmid trimethoprim resistance.* J Biol Chem 1986;261:2503-2505.

CHAPTER 7

NUCLEOTIDE SEQUENCE OF A TRIMETHOPRIM-RESISTANT CHROMOSOMALLY ENCODED DIHYDROFOLATE REDUCTASE FROM HAEMOPHILUS INFLUENZAE

Manuscript submitted.

CHAPTER 7

NUCLEOTIDE SEQUENCE OF A TRIMETHOPRIM-RESISTANT CHROMOSOMALLY ENCODED DIHYDROFOLATE REDUCTASE FROM HAEMOPHILUS INFLUENZAE

Ronald de Groot[1], Marcel Sluijter[1], Arnold L. Smith[2], Wil H.F. Goessens[3]

[1] *Department of Pediatrics, Sophia Children's Hospital and Erasmus University, Rotterdam, The Netherlands.*
[2] *Department of Pediatrics, University of Washington, Seattle, USA.*
[3] *Department of Microbiology, University Hospital Dijkzigt and Erasmus University, Rotterdam, The Netherlands.*

7.1 SUMMARY

Trimethoprim resistance in *Haemophilus influenzae* (*H. influenzae*) is due to overproduction of altered dihydrofolate reductase(s) (DHFR(s)). The nucleotide sequence of a 1144 bp *H. influenzae* DNA fragment, which confers resistance to trimethoprim was determined. An open reading frame (ORF) of 480 bp was identified, which encoded a polypeptide with a predicted molecular mass of 17760 Da. The deduced amino acid sequence shows significant homology with several prokaryotic and eukaryotic DHFRs. The upstream region contains sequences homologous to prokaryotic promoters and has a putative ribosome binding site. The nucleotide sequence 5' of the DHFR gene contains a 561 bp ORF without a termination codon. The gene encoded is transcribed in the opposite direction and the deduced protein has 62.9% homology with the N-terminal sequence of the *E. coli* proBA region encoding γ-glutamyl-kinase.

7.2 INTRODUCTION

Dihydrofolate reductase (tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. Tetrahydrofolate serves at different oxidation levels as a carrier of one-carbon units for the biosynthesis of purines, pyrimidines, methionine and serine [6]. Several folate analogues including the antibiotic trimethoprim (Tmp) and the antineoplastic drug methotrexate competitively inhibit prokaryotic and/or eukaryotic dihydrofolate reductase (DHFR) resulting in cessation of growth followed by cell death. The selective activity of Tmp [2,4-diamino-5-(3',4',5'-trimethoxybenzyl) pyrimidine] on prokaryotic cells is based on a several thousand-fold higher affinity of Tmp for bacterial DHFRs, than for mammalian DHFRs [8]. X-ray diffraction studies indicate, that binding of folate analogues to the catalytic site of DHFR occurs in a hydrophobic binding pocket, which contains 4 amino acid residue regions [24, 26].

Trimethoprim-resistant bacteria were reported shortly after its introduction in the 1960s. The major mechanism of resistance is based upon production of plasmid-encoded altered DHFRs [1]. Resistance due to decreased cell wall permeability, thymine auxotrophy, chromosomal mutations in promoter sequences, ribosome binding sites or the structural gene for DHFR ("fol") leading to overproduction of DHFR or a decreased substrate affinity are less common.

We previously reported the molecular cloning of a trimethoprim resistance (Tmp^r) gene from *H. influenzae* [16]. Southern blots using an intragenic probe indicated, that the Tmp^r gene in *H. influenzae* showed no homology with Tmp^r encoding genes in a variety of Gram-negative bacteria [16]. Investigation of Tmp^r *H. influenzae* isolates subsequently indicated, that the mechanism of resistance in these strains is caused by overproduction of structurally altered DHFRs [17]. In order to clarify the genetic basis of Tmp resistance we decided to subclone and sequence the gene encoding Tmp resistance of *H. influenzae* in strain R1047. We here report the nucleotide sequence of a 1144 bp *H. influenzae* fragment, which confers resistance to Tmp. Two open reading frames (ORFs) of respectively 480 and 561 bp were identified. The 480 bp ORF specified a protein with a predicted molecular mass of 17760 Da. The nucleotide and promoter sequences and the ribosome binding site showed significant homology with several Tmp^s and Tmp^r DHFR sequences from both eukaryotes and prokaryotes. The nucleotide sequence 5' of the DHFR gene contains an ORF with 62.9% homology with the *E. coli* proBA operon encoding γ-glutamyl-kinase. The proBA gene is transcribed in the opposite direction and preceded by putative promoter and ribosome binding sites.

7.3 MATERIALS AND METHODS

Bacterial strains, DNA cloning vectors and growth conditions

The *E. coli* K12 strain HB101 [7] was used as a host for the pGEM3 derivative pRGS7, which carries a 12.9-kb insert coding for Tmp resistance [16]. Plasmid subcloning was accomplished using pTZ18 or 19 plasmids [27]. *E. coli* strains were grown aerobically at 37°C, either in Luria broth [32] with shaking (200 r.p.m.) or on Luria agar [Luria broth plus 1.5% Bacto-agar (Difco Laboratories, Detroit, Mich)] or 1.5% Agar technical (Oxoid, Basingstoke, U.K.). Unless otherwise stated, Tmp (obtained from Sigma Chemical Co., St. Louis, Mo) and ampicillin (purchased from Gist-Brocades, Delft, The Netherlands) were added to broth or solid media at a final concentration of 5 μg/ml and 100 μg/ml respectively.

Antimicrobial susceptibility

Determinations of the MIC of Tmp for pRGS12 and pTZ18/19 were performed by agar dilution [25]. Bacteria were inoculated on Haemophilus test medium [23] plus 0.2 units thymidine phosphorylase/ml containing Tmp at graded concentrations between 0.12 and 128 μg/ml. The inocula tested were 10^4 and 10^5 CFU. The MIC was defined as the lowest concentration, which completely inhibited visible growth in comparison with growth on antibiotic-free medium.

DNA techniques

Small-scale plasmid DNA was prepared as described by Birnboim and Doly [5]. The methods of restriction endonuclease digestion, DNA fragment ligation and transformation of plasmid DNA into *E. coli* were carried out by standard procedures [32]. DNA was digested and electrophoresed on low-melting-temperature agarose gels for preparation of fragments. DNA fragments were stained with ethidiumbromide, visualized by UV light and the bands of interest were excised. Fragments were purified by means of a Geneclean kit (BIO 101, La Jolla, Ca) and subsequently used for ligation. All restriction endonucleases, ATP and T4 DNA ligase were obtained from Boehringer GmbH (Mannheim, FRG) and used in accordance with the instructions of the manufacturers. Recombinant plasmids containing DNA fragments from pRGS7 in pTZ18 or 19 were detected on plates containing 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal, supplied by Sigma) and 5mM isopropyl-β-D-thiogalactopyranoside (IPTG, obtained from Sigma). Five restriction enzyme fragments were subcloned from pRGS7 [16]. These subclones are all derived from a 2.2-kb *Xba*I-*Eco*RI fragment, which was previously shown in transformation experiments to contain the gene encoding Tmp resistance.

Nucleotide sequencing and oligonucleotide synthesis

Nucleotide sequencing of an approximately 1.6-kb *Xba*I-*Eco*RI fragment of pRGS7 in pTZ18 and 19, which confers Tmp resistance in *E. coli* HB101, was carried out on both strands by the dideoxy nucleotide chain termination method [33]. For optimal results T7 DNA polymerase (Promega, Leiden, The Netherlands) was used in combination with the Sequenase kit of US Biochemicals (Cleveland, Oh, USA). The Taq DNA polymerase kit from Promega was used to overcome band compressions. Double stranded closed circular plasmid templates were used as described by Chen et al. [11]. Nicktranslation of plasmid DNA was performed with [α-^{35}S]dATP (Amersham, United Kingdom). Universal primers (17 mer) or oligonucleotide internal primers (20 mer) were used for priming the dideoxy chain-termination reactions. Oligonucleotide primers were synthesized on an Applied Biosystems 381A DNA synthesizer using the β-cyanoethyl phosphoramidite method. Computer analysis of nucleic acid sequences was carried out using a Microgenie software package (Beckman, Palo Alto, Ca) on a Tulip Compact 2 computer. Comparisons between different nucleotide and amino acid sequences were performed using the Fasta program from caos/camm and the facilities of the Department of Genetics, Erasmus University, Rotterdam.

Nucleotide sequence accession number

A 1144 bp region encoding trimethoprim-resistant dihydrofolate reductase from strain R1047 is EMBL accession number X59128.

7.4 RESULTS AND DISCUSSION

Identification of a DNA fragment containing the dihydrofolate reductase (DHFR) gene

The Tmp^r *E. coli* strain HB101 containing plasmid pRGS7 was used for subcloning [16]. Previous experiments indicated, that transformation to Tmp resistance with a 2.2-kb *Hin*fI fragment of pRGS7 was possible at high frequencies. However, Tmp^s *H. influenzae* isolates could not be transformed to Tmp resistance with a 0.50-kb *Xba*I-*Pst*I fragment, which is internal to the *Hin*fI DNA fragment. Five subclones were obtained as indicated in Figure 7.1. The potential expression of Tmp resistance of the subclones (pRGS8, 9, 10, 11 and 12) in *E. coli* HB101 was confirmed on Iso-Sensitest agar plates containing a Tmp concentration of 5 μg/ml. Strains containing pRGS8 and pRGS12 encoded Tmp resistance, whereas the other three isolates (pRGS9, 10 and 11) were Tmp-susceptible. This confirmed our previous findings, that the gene encoding Tmp resistance is predominantly located on the 0.50-kb *Xba*I-*Pst*I fragment. The agar dilution MIC of pRGS12 was 128

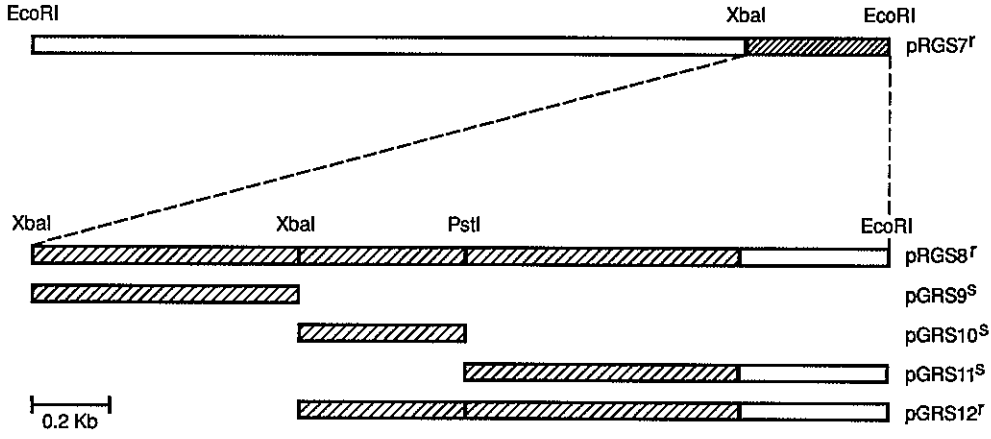

Figure 7.1 Restriction endonuclease map of pRGS7 [16] and five subclones in pTZ18/19.

Abbreviations: r indicates expression of resistance to trimethoprim, s indicates susceptibility to trimethoprim. The open bars in pRGS8, 11 and 12 represent a subcloned sequence from pGEM3.

μg/ml, which is significantly higher than the MIC of strain R1047 (40 μg/ml), from which the cloned *H. influenzae* DNA was derived. The higher MIC may be caused through overproduction of DHFR by multiple copies of pRGS12 in *E. coli* HB101.

DNA sequence analysis of the 1144 bp fragment

The approximately 1.6-kb subclone pRGS12 was completely sequenced on both strands using universal and several synthetic oligonucleotide primers. The first 383 nucleotides 5' from the *Eco*RI site were identical to the pGEM3 sequence, which was used for subcloning. The remaining 1144 bp sequence contained two ORFs shown in Figure 7.2. The first ORF (Figure 7.2a) starting at nucleotide 634, encodes a protein of 160 amino acid residues, and terminates at TAA (nucleotides 1114 to 1116). The start codon is preceded by a potential ribosome binding site [35] and by -10 [28] and -35 [29] promoter regions. The ORF encodes a protein with a predicted molecular mass of 17.760 kDa.
The second ORF is situated on the complementary DNA strand in an orientation opposite to the first ORF and has a start codon at position 523. This ORF does not contain a termination codon indicating that only the N-terminal sequence of the gene has been cloned. Its translational initiation codon and expected putative promoter and ribosome binding sites are indicated in Figure 7.2b.

```
#####
GATCACTATCAAATAAACCTTGTTGATCAGTTAATAAATAAAGTTGTTCCGCTTGAACAAGAATAGCGACTAATGCAGATAAATTGTCATTATCGCCAAC    100

TTTAATTTCTGCGGTTGCCACCGCATCATTTTCATTAATCACAGGAATAATATGGTTATCTAAAAGTGCATGTAAAGTATCTCGCGCATTTAAAAAACGT    200

TCGCGATCTTCAATATCAGCACGAGTTAATAAAAGTTGTCCGATGTGAATATCATAAATAGCAAATAATTTTTCCCAAGCCTGAATTAATTGGCTCTGAC    300

CAACTGCTGCAAGCAGTTGCTTTGAAGCAATAGTGGGTGGTAATTGAGGATGATTTAAATAATGGCGACCCGCAGCAATGGCACCAGAAGTCACGATCAC    400

TATACGAAATCCATCATTATGCAGTTGTGCAATTTGACGAACGATTTCCATCATGTGCGGTGAATTTAGTTTTGGTGAACCCTGTGTTAGAGTACTTGTA    500

CCAAATTTCACTACGATTGTTTTCTTGTTCATAGTCGTTTCCAAAGTTCTTAAAATTGACATTAACGTTATCACTAAATCAATGACAAATCACTTAATAT    600

CAGGTATAGTAACGGCAAATTTTAGGGGGACTT   ATG ACA TTT AGT TTA ATT GTA GCG ACG ACA TTA AAT AGT GTA ATT GGT    681
                 • ••• ••          met thr phe ser leu ile val ala thr thr leu asn ser val ile gly
                                  PstI
AAA GAT AAC CAA ATT CCT TGG CAC TTG CCT GCA GAT TTA GCT TGG TTT CGT CAG AAC ACC ACT GGT AAA CCT GTC    756
lys asp asn gln ile pro trp his leu pro ala asp leu ala trp phe arg gln asn thr thr gly lys pro val

ATT ATG GGG CGT AAA ACC TTT GAA AGT ATT GGT CGT GCA CTA CCT AAA CGT ACC AAT ATC GTA CTT TCT CGC CAG    831
ile met gly arg lys thr phe glu ser ile gly arg ala leu pro lys arg thr asn ile val leu ser arg gln

CTT TTT GAA CAC GAA GGT GTG ATA TGG AAA GAT AGC TTT GAA AGT GCG GTC AAT TTT GTC AGA GAT TTT GAT GAA    906
leu phe glu his glu gly val ile trp lys asp ser phe glu ser ala val asn phe val arg asp phe asp glu

ATT ATG TTG CTT GGT GGG GGA GAG TTA TTC AAA CAA TAT TTA CCC AAA GCA GAT AAG TTA TAC CTT ACT CAA ATT    981
ile met leu leu gly gly gly glu leu phe lys gln tyr leu pro lys ala asp lys leu tyr leu thr gln ile

CAA ACA GAA CTA GAT GGT GAT ACT TTT TTC CCT CAA TTG AAT TGG GAG GAG TGG GAA ATT GAA TTT GAT GAA TAT   1056
gln thr glu leu asp gly asp thr phe phe pro gln leu asn trp glu glu trp glu ile glu phe asp glu tyr

CGT AAG GCG GAT GAA CAA AAT CGC TAT GAT TGC CGA TTT TTA ATT CTT ACC CGA AAA TAA ATCAAATAAATGAAACAATA   1136
arg lys ala asp glu gln asn arg tyr asp cys arg phe leu ile leu thr arg lys ***
  XbaI
CTTCTAGA                                                                                              1144
```

Figure 7.2a Nucleotide sequence of the 1144 bp DNA fragment containing the DHFR gene from *H. influenzae* R1047. The sequence is written in the 5' - 3' direction of the coding strand with the deduced amino acid sequence of the DHFR gene below. Potential -10 and -35 promoter sequences are underlined. Homology between the putative ribosome binding site and the consensus *E. coli* 16S rRNA polymerase sequence is indicated with dots. The intragenic *PstI* site is marked.

Identification of the gene encoding dihydrofolate reductase

Computer analysis of the nucleotide and deduced amino acid sequences from the first ORF revealed significant homology with prokaryotic and eukaryotic DHFRs. The 160 amino acid DHFR from *H. influenzae* strain R1047 showed 51.3% homology in an overlap of 158 amino acids with the DHFR from *E. coli* K12 [37]. The molecular mass of *H. influenzae* DHFR (17.760 kDa) was similar to the molecular mass of other prokaryotic DHFRs (approximately 18 kDa) and approximates the calculated Mr of purified DHFR [17]. The homology with other prokaryotic Tmp^s and Tmp^r DHFRs varied between 34.9% and 49.1% (Figure 7.3). As expected, homology with eukaryotic DHFRs was significantly less and mainly present in the first 68 sequences of the N-terminal site of the protein. Comparison between the Tmp^r DHFR from *H. influenzae* R1047 and DHFRs from Tmp^s and Tmp^r prokaryotes was performed using the alignment of amino acid sequences based on structural information from X-ray crystallography [45]. Fifteen amino acid residues are highly conserved among all Tmp^r and Tmp^s DHFRs (Figure 7.3). These residues are predominantly located in the region of the enzyme forming the hydrophobic binding pocket [24] which has been implicated in the interaction of DHFR with its cofactor and/or inhibitors [45]. The structural and functional differences between DHFR from Tmp^r

```
 XbaI
TCTAGAAGTATTGTTTCATTTATTTGATTTATTTTCGGGTAAGAATTAAAAATCGGCAATCATAGCGATTTTGTTCATCCGCCTTACGATATTCATCAAA  1045

TTCAATTTCCCACTCCTCCCAATTCAATTGAGGGAAAAAAGTATCACCATCTAGTTCTGTTTGAATTTGAGTAAGGTATAACTTATCTGCTTTGGGTAAA   945

TATTGTTTGAATAACTCTCCCCCACCAAGCAACATAATTTCATCAAAATCTCTGACAAAATTGACCGCACTTTCAAAGCTATCTTTCCATATCACACCTT   845

CGTGTTCAAAAAGCTGGCGAGAAAGTACGATATTGGTACGTTTAGGTAGTGCACGACCAATACTTTCAAAGGTTTTACGCCCCATAATGACAGGTTTACC   745
                                  PstI
AGTGGTGTTCTGACGAAACCAAGCTAAATCTGCAGGCAAGTGCCAAGGAATTTGGTTATCTTTACCAATTACACTATTTAATGTCGTCGCTACAATTAAA   645

CTAAATGTCATAAGTCCCCCTAAAATTTGCCGTTACTATACCTGATATTAAGTGATTTGTCATTGATTTAGTGATAACGTTAATGTCAATTTTAAGAACT   545
                                                                                          ****  *
TTGGAAACGACT ATG AAC AAG AAA ACA ATC GTA GTG AAA TTT GGT ACA AGT ACT CTA ACA CAG GGT TCA CCA AAA CTA      467
*            met asn lys lys thr ile val val lys phe gly thr ser thr leu thr gln gly ser pro lys leu

AAT TCA CCG CAC ATG ATG GAA ATC GTT CGT CAA ATT GCA CAA CTG CAT AAT GAT GGA TTT CGT ATA GTG ATC GTG      392
asn ser pro his met met glu ile val arg gln ile ala gln leu his asn asp gly phe arg ile val ile val

ACT TCT GGT GCC ATT GCT GCG GGT CGC CAT TAT TTA AAT CAT CCT CAA TTA CCA CCC ACT ATT GCT TCA AAG CAA      317
thr ser gly ala ile ala ala gly arg his tyr leu asn his pro gln leu pro pro thr ile ala ser lys gln

CTG CTT GCA GCA GTT GGT CAG AGC CAA TTA ATT CAG GCT TGG GAA AAA TTA TTT GCT ATT TAT GAT ATT CAC ATC      242
leu leu ala ala val gly gln ser gln leu ile gln ala trp glu lys leu phe ala ile tyr asp ile his ile

GGA CAA CTT TTA TTA ACT CGT GCT GAT ATT GAA GAT CGC GAA CGT TTT TTA AAT GCG CGA GAT ACT TTA CAT GCA      167
gly gln leu leu leu thr arg ala asp ile glu asp arg glu arg phe leu asn ala arg asp thr leu his ala

CTT TTA GAT AAC CAT ATT ATT CCT GTG ATT AAT GAA AAT GAT GCG GTG GCA ACC GCA GAA ATT AAA GTT GGC GAT       92
leu leu asp asn his ile ile pro val ile asn glu asn asp ala val ala thr ala glu ile lys val gly asp

AAT GAC AAT TTA TCT GCA TTA GTC GCT ATT CTT GTT CAA GCG GAA CAA CTT TAT TTA TTA ACT GAT CAA CAA GGT       17
asn asp asn leu ser ala leu val ala ile leu val gln ala glu gln leu tyr leu leu thr asp gln gln gly
                ####
TTA TTT GAT AGT GAT C                                                                                    1
leu phe asp ser asp
```

Figure 7.2b Nucleotide sequence of 1144 bp sequence above on the complementary strand with the reading frame in the opposite direction. The deduced amino acid sequence of the N-terminal site of the γ-glutamyl-kinase gene is indicated below. Potential -10 and -35 promoter sequences are underlined. Homology between the putative ribosome binding site and the consensus *E.coli* 16S rRNA polymerase sequence is indicated with dots.

and Tmp^s *H. influenzae* strains suggest the presence of (a) mutation(s) in the "fol" gene. Several studies have indicated, that a single nucleotide change in conserved sequences of the structural "fol" gene may result in major changes in inhibitor binding and kinetic properties of DHFR [2, 10, 22, 39, 44]. Comparison between *H. influenzae* R1047 DHFR and other DHFRs indicates, that the highly conserved glutamate residue in Tmp^s DHFR at position 121 may have converted into aspartate. The significance of the substitution at this position needs to be confirmed by comparison with the DHFR sequence from Tmp^s *H. influenzae*.

The alignment between the promoter region of *H. influenzae* R1047 DHFR and the consensus *E. coli* promoter indicates, that the -10 sequence is completely homologous to the *E. coli* K12 DHFR promoter sequence [TATAGT, 29], whereas the -35 sequence is identical in 4 of 6 nucleotides to the -35 region of *E. coli* K12 DHFR [TCGACA, 37]. The -35 sequence is identical in 5 of 6 nucleotides to the -35 region of *E. coli* 1810 DHFR [TTGACA], which overproduces DHFR several hundredfold [12]. The consequences of changes in the -35 region for overproduction of DHFR have also been reported by Smith et al. [39], who showed that a C to T transversion in position -34 may increase the synthesis of "fol" mRNA by 12 to 15-fold. This level of overproduction is in a similar range as the overproduction of DHFR in Tmp^r DHFR. We therefore speculate, that changes in

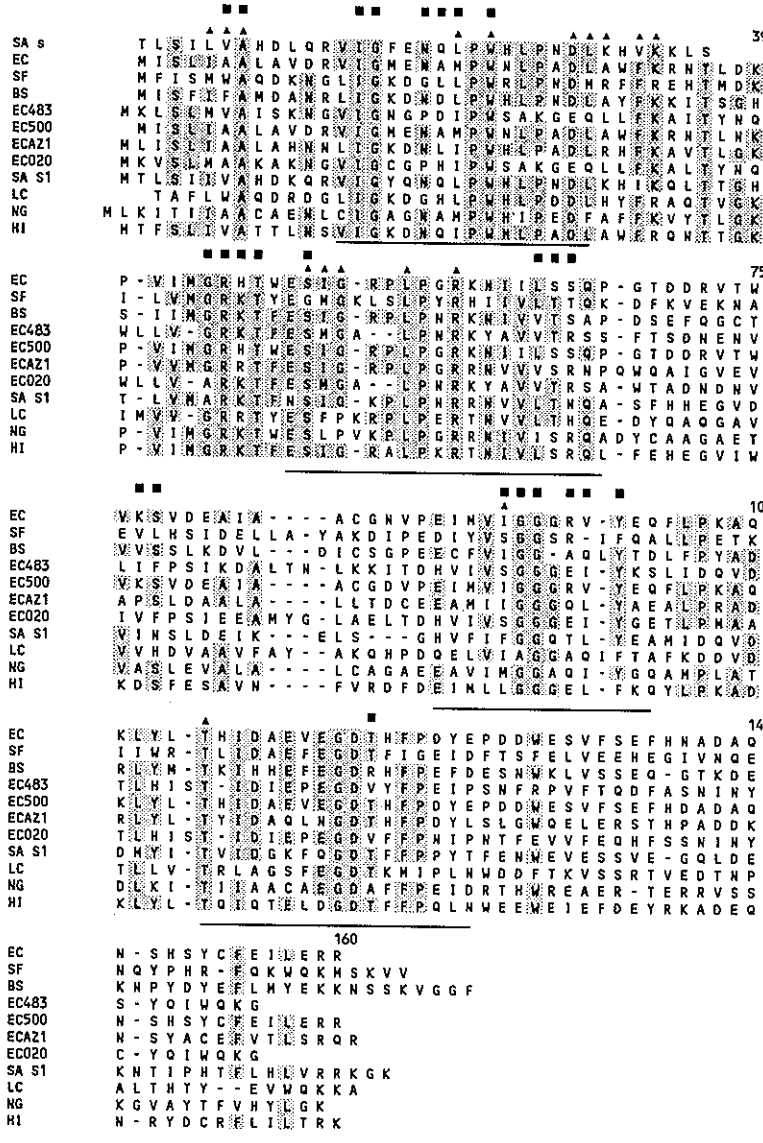

Figure 7.3 Comparison of the *H. influenzae* DHFR with several prokaryotic DHFRs.

The sequences are aligned as described by Volz et al. [45]. Abbreviations: SA s, *Staphylococcus aureus* [19], EC, *Escherichia coli* K12 [37], SF, *Streptococcus faecium* [15], BS, *Bacillus subtilis* [22], EC 483, *E.coli* type I DHFR [36], EC500, *E. coli* 500 mutant strain [42], EC AZ1, *E.coli* type III DHFR [13], EC 020, *E.coli* type V DHFR [43], SAS1 *Staphylococcus aureus* type I DHFR [30], LC *Lactobacillus casei* [14], NG, *Neisseria gonorrhoeae* [3], HI, *Haemophilus influenzae* R1047. Symbols: ▲ NADPH-binding site, ■ inhibitor-binding site. The regions of the enzyme forming the hydrophobic binding pocket are underlined. Positions at which the *H. influenzae* enzyme are identical to six or more amino acids of the other DHFRs are boxed.

the -35 and/or -10 region similar to those seen in *E. coli* mutant strains may be responsible for overproduction of *H. influenzae* DHFR in strain R1047. It would be interesting to assess by oligonucleotide site-directed mutagenesis if a conversion from A to T in the -35 position of *H. influenzae* R1047 DHFR resulting in a complete homology with the TTG sequence, characteristic for strong Gram-negative promoters [18], will result in a further significant increase in the overproduction of DHFR. Chang et al. and Flensburg et al. have shown, that the efficiency of translation is also strongly affected by the extent of homology of the translational control region with the 3' end of 16S rRNA and by the distance between the protein start codon and the ribosome binding sequence (RBS) in mRNA [9, 12]. Further analysis of the RBS from the Tmp^s and highly Tmp^r *H. influenzae* DHFRs will be performed to determine if significant changes in these sequences may also contribute to the overproduction of DHFR in Tmp^r *H. influenzae*.

Identification of the gene encoding γ-glutamyl-kinase

The ORF 5' of the DHFR gene on the complementary strand is transcribed in the opposite direction and contains 561 nucleotides (Figure 7.2b). Computer analysis of this ORF indicated 62.9% identity with the first 187 amino acids of the N-terminal sequence of the *E. coli* proBA region. The proB gene encodes γ-glutamyl-kinase (ATP:L-glutamate-5-phosphotransferase, EC 2.7.2.11), which activates the γ-carboxyl group of glutamate to γ-glutamyl-phosphate [40]. The proA gene, which is part of the same operon in *E. coli* encodes NADPH-dependent glutamate semialdehyde dehydrogenase, which catalyzes γ-glutamyl-phosphate into glutamate γ-semialdehyde [20]. It has been suggested, that these two enzymes function as an enzyme complex [34], which catalyzes the first steps in the pathway from glutamate to proline. Proline has an important role in bacterial protein synthesis and serves as a carbon and nitrogen source. It may also act as a protectant against osmotic stress. Mutations in the proBA operon may lead to overproduction of proline and osmotic tolerance due to a decreased allosteric feedback inhibition of γ-glutamyl-kinase by proline [31, 41]. It is interesting, that the proBA map position in *E. coli* (at 6 min) is far removed from the DHFR "fol A" gene (at 1 min) [4], whereas these two genes are clustered in the *H. influenzae* genome. Analysis of the region upstream from the 5'-terminus of the proB gene indicates the presence of a putative RBS and -10 and -35 promoter sequences (Figure 7.2b).

Acknowledgements

We are indebted to Jacqueline Dito for typing the manuscript, to Sjozef van Baal for support with the nucleotide sequence analysis, and to Bert Niesters for oligonucleotide synthesis. This work was supported by the Sophia Foundation for Scientific Research.

7.5 REFERENCES

1 **Amyes S.G.B., Towner K.J.** *Trimethoprim resistance; epidemiology and molecular aspects.* J Med Microbiol 1990;31:1-19.

2 **Baccanari D.P., Stone D., Kuyper L.** *Effect of a single amino acid substitution on Escherichia coli dihydrofolate reductase catalysis and ligand binding.* J Biol Chem 1981;256:1738-1747.

3 **Baccanari D.P., Tansik R.L., Paterson R.J., Stone D.** *Characterization and amino acid sequence of Neisseria gonorrhoeae dihydrofolate reductase.* J Biol Chem 1984;259:12291-12298.

4 **Bachmann B.J.** *Linkage map of Escherichia coli K-12, Edition 8.* Microbiol Rev 1990;54:130-197.

5 **Birnboim H.C., Doly J.** *A rapid alkaline extraction procedure for screening recombinant plasmid DNA.* Nucleic Acids Res 1979;7:1513-1523.

6 **Blakley R.L.** *The biochemistry of folic acid and related pteridines.* North-Holland Publishing Co. Amsterdam 1969:139-187.

7 **Boyer H.W., Roulland-Dussoix D.** *A complementation analysis of the restriction and modification of DNA in Escherichia coli.* J Mol Biol 1969;41:459-472.

8 **Burchall J.J.** *Comparative biochemistry of dihydrofolate reductase.* Ann N Y Acad Sci 1971;186:143-152.

9 **Chang A.C.Y., Erlich H.A., Gunsalus R.P., Nunberg J.H., Kaufman R.J., Schimke R.T., Cohen S.N.** *Initiation of protein synthesis in bacteria at a translational start codon of mammalian cDNA: effects of the preceding nucleotide sequence.* Proc Natl Acad Sci USA 1980;77:1442-1446.

10 **Chen J.T., Mayer R.J., Flerke C.A., Benkovic S.J.** *Site-specific mutagenesis of dihydrofolate reductase from Escherichia coli.* J Cell Biochem 1985;29:73-82.

11 **Chen E.Y., Seeburg P.H.** *Supercoil sequencing: a fast and simple method for sequencing plasmid DNA.* DNA 1985;4:165-170.

12 **Flensburg J., Skōld O.** *Massive overproduction of dihydrofolate reductase in bacteria as a response to the use of trimethoprim.* Eur J Biochem 1987;162:473-476.

13 **Fling M.E., Kopf J., Richards C.** *Characterization of plasmid pAZ1 and the type III dihydrofolate reductase gene.* Plasmid 1988;19:30-38.

14 **Freisheim J.H., Bitar K.G., Reddy A.V., Blankenship D.T.** *Dihydrofolate reductase from amethopterin-resistant Lactobacillus casei.* J Biol Chem 1978;253:6437-6444.

15 **Gleisner J.M., Peterson D.L., Blakley R.L.** *Amino acid sequence of dihydrofolate reductase from a methotrexate-resistant mutant of Streptococcus faecium and identification of methionine residues at the inhibitor binding site.* Proc Natl Acad Sci USA 1974;71:3001-3005.

16 **de Groot R., Campos J., Moseley S.L., Smith A.L.** *Molecular cloning and mechanism of trimethoprim resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1988;32:477-484.

17 **de Groot R., Chaffin D.O., Kuehn M., Smith A.L.** *Trimethoprim resistance in Haemophilus influenzae is due to altered dihydrofolate reductase(s).* Biochem J 1991;274:657-662.

18 **Harley C.B., Reynolds R.P.** *Analysis of E. coli promoter sequences.* Nucleic Acids Res 1987;15:2343-2361.

19 **Hartman P.G., Ståhl M., Kocher H.P., Then R.L.** *N-terminal amino acid sequence of the chromosomal dihydrofolate reductase purified from trimethoprim-resistant Staphylococcus aureus.* FEBS Lett 1988;242:157-160.

20 **Hayzer D.J., Leisinger T.** *Proline biosynthesis in Escherichia coli.* Eur J Biochem 1982;121:561-565.

21 **Hung A., Thillet J., Pictet R.** *In vivo selected promoter and ribosome binding site up-mutations: demonstration that the Escherichia coli bla promoter and a Shine-Dalgarno region with low complementarity to the 16S ribosomal RNA function in Bacillus subtilis.* Mol Gen Genet 1989;219:129-136.

22 **Iwakura M., Kawata M., Tsuda K., Tanaka T.** *Nucleotide sequence of the thymidylate synthase B and dihydrofolate reductase genes contained in one Bacillus subtilis operon.* Gene 1988;64:9-20.

23 **Jorgensen J.H., Redding J.S., Maher L.A., Howel A.W.** *Improved medium for antimicrobial susceptibility testing of Haemophilus influenzae.* J Clin Microbiol 1987;25:2105-2113.

24 **Kellems R.E., Alt F.W., Schimke R.T.** *Regulation of folate reductase synthesis in sensitive and methotrexate-resistant sarcoma 180 cells.* J Biol Chem 1976;251:6987-6993.

25 **Lennette E.H., Balows A., Hausler W.J., Shadomy H.J.** *Manual of clinical microbiology, 4th ed.* American Society for Microbiology, Washington D.C.;1985.

26 **Matthews D.A., Alden R.A., Bolin J.T., Freer S.T.** *Dihydrofolate reductase: X-ray structure of the binary complex with methotrexate.* Science 1977;197:452-455.

27 **Mead D.A., Szczesna-Skorupa E., Kemper B.** *Single-stranded DNA blue T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering.* Prot Engineer 1986;1:67-74.

28 **Pribnow D.** *Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter.* Proc Nat Acad Sci USA 1975;72:784-788.

29 **Rosenberg M., Court D.** *Regulatory sequences involved in the promotion and termination of RNA transcription.* Ann Rev Genet 1979;13:319-353.

30 **Rouch D.A., Messerotti L.J., Loo L.S.L., Jackson C.A., Skurray R.A.** *Trimethoprim resistance transposon Tn4003 from Staphylococcus aureus encodes genes for a dihydrofolate reductase and thymidylate synthetase flanked by three copies of IS257.* Mol Microbiol 1989;3:161-175.

31 **Rushlow K.E., Deutch A.H., Smith C.J.** *Identification of a mutation that relieves γ-glutamyl-kinase from allosteric feedback inhibition by proline.* Gene 1984;39:109-112.

32 **Sambrook J., Fritsch E.F., Maniatis T.** *Molecular cloning: a laboratory manual.* Cold Spring Harbor Laboratory Press 1989.

33 **Sanger F., Nicklen S., Coulsen A.R.** *DNA sequencing with chain-terminating inhibitors.* Proc Natl Acad Sci USA 1977;74:5463-5467.

34 **Seddon A.P., Zhao K.Y., Meister A.** *Activation of glutamate by γ-glutamate-kinase: formation of γ-cis-cycloglutamyl-phosphate, an analog of γ-glutamyl-phosphate.* J Biol Chem 1989;264:11326-11335.

35 **Shine J., Dalgarno L.** *The 3'-terminal sequence of Escherichia coli 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites.* Proc Nat Acad Sci USA 1974;71:1342-1346.

36 **Simonsen C.C., Chen E.Y., Levinson A.D.** *Identification of the type I trimethoprim-resistant dihydrofolate reductase specified by the Escherichia coli R-plasmid R483: comparison with procaryotic and eucaryotic dihydrofolate reductases.* J Bacteriol 1983;155:1001-1008.

37 **Smith D.R., Calvo J.M.** *Nucleotide sequence of the E. coli gene coding for dihydrofolate reductase.* Nucleic Acids Res 1980;8:2255-2274.

38 **Smith D.R., Calvo J.M.** *Nucleotide sequence of dihydrofolate reductase genes from trimethoprim-resistant mutants of Escherichia coli.* Mol Gen Genet 1982;187:72-78.

39 **Smith D.R., Rood J.I., Bird P.I., Sneddon M.K., Calvo J.M., Morrison J.F.** *Amplification and modification of dihydrofolate reductase in Escherichia coli.* J Biol Chem 1982;257:9043-9048.

40 **Smith C.J., Deutch A.H., Rushlow K.E.** *Purification and characteristics of a γ-glutamyl-kinase involved in Escherichia coli proline biosynthesis.* J Bacteriol 1984;157:545-551.

41 **Smith L.T.** *Characterization of a γ-glutamyl-kinase from Escherichia coli that confers proline overproduction and osmotic tolerance.* J Bacteriol 1985;164:1088-1093.

42 **Stone D., Phillpps A.W., Burchall J.J.** *The amino acid sequence of the dihydrofolate reductase EC 1.5.1.3 of a trimethoprim-resistant strain of Escherichia coli.* Eur J Biochem 1977;72:613-624.

43 **Sundström L., Rådström P., Swedberg G., Sköld O.** *Site-specific recombination promotes linkage between trimethoprim- and sulfonamide resistance genes. Sequence characterization of dhfrV and sulI and a recombination active locus of Tn21.* Mol Gen Genet 1988;213:191-201.

44 **Villafranca J.E., Howell E.E., Voet D.H., Strobel M.S., Ogden R.C., Abelson J.N., Kraut J.** *Directed mutagenesis of dihydrofolate reductase.* Science 1983;222:782-788.

45 **Volz K.W., Matthews D.A., Alden R.A., Freer S.T., Hansch C., Kaufman B.T., Kraut J.** *Crystal structure of avian dihydrofolate reductase containing phenyltriazine and NADPH.* J Biol Chem 1982;257:2528-2536.

CHAPTER 8

GENETIC RELATEDNESS OF ANTIBIOTIC RESISTANCE DETERMINANTS IN MULTIPLE-RESISTANT HAEMOPHILUS INFLUENZAE

J Infect Dis 1989;160:810-817

CHAPTER 8

GENETIC RELATEDNESS OF ANTIBIOTIC RESISTANCE DETERMINANTS IN MULTIPLE-RESISTANT HAEMOPHILUS INFLUENZAE

José Campos, Monchai Chanyangam, Ronald de Groot, Arnold Smith, Fred Tenover, Roser Reig.

Division of Infectious Disease, Children's Hospital and Medical Center, Seattle, U.S.A.
Department of Microbiology, Hospital Infantil San Juan de Dios, Barcelona, Spain.
Department of Pediatrics and Laboratory Medicine, University of Washington, Seattle, U.S.A.
Laboratory Service, Veterans Administration Medical Center, Seattle, U.S.A.
Facultad de Medicina, Departamento de Microbiologia, Universidad Autonoma de Barcelona, Spain.

8.1 SUMMARY

Antimicrobial resistance determinants and plasmids present in 10 multiple antibiotic-resistant strains of *Haemophilus influenzae* isolated from patients in different geographic regions of Spain were characterized. Conjugative plasmids with molecular sizes of 38-50 MDa encoded resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, sulfamethoxazole, and tetracycline. Trimethoprim resistance was not linked to the other antibiotic resistance determinants and trimethoprim-resistant transconjugants and transformants lacked detectable plasmid DNA, suggesting that this determinant is chromosomal. Restriction endonuclease analysis revealed similarities among the plasmids, but several restriction patterns could be distinguished. Three hybridization patterns were found with DNA probes coding for *H. parainfluenzae* β-lactamase and chloramphenicol-acetyltransferase. Resistance to kanamycin was due to drug modification by aminoglycoside-phosphotransferase(3')1. In Spain, it appears that multiple antibiotic resistance

phenotypes in *H. influenzae* did not arise from acquisition of a single R-plasmid; rather, both plasmid and chromosomal resistance evolved independently from several sources.

8.2 INTRODUCTION

Strains of *Haemophilus influenzae* type b resistant to ampicillin and chloramphenicol have been reported since 1979 [1, 17, 22, 31, 37]. We have previously shown that strains resistant to these antibiotics and tetracycline, with or without resistance to trimethoprim, kanamycin, sulfamethoxazole, and streptomycin, are prevalent in Spain [3, 4]. The genes encoding resistance to ampicillin and chloramphenicol are present on high-molecular-weight conjugative plasmids [19, 22, 30, 31, 34]. However, it is not clear whether the R-plasmids in these multiple-resistant isolates rose independently or were due to the dissemination of a single plasmid clone. Further, it was not known whether the antibiotic-resistant genes were identical or dissimilar. We, therefore, characterized the genetic basis of the multiple antimicrobial resistance determinants present in *H. influenzae* isolated in Spain.

8.3 MATERIALS AND METHODS

Bacterial strains.

Ten multiple-resistant strains were studied that were isolated in different geographic areas of Spain (Table 8.1). No epidemiologic link could be established between the strains [3, 4]. *H. influenzae* was identified by standard methods [18]; eight were type b by slide agglutination with Difco Laboratories (Detroit) antisera, whereas two were untypeable with Difco polyvalent antisera. Strain MAP was derived by single-step resistance to streptomycin at 250 μg/ml [7].

Media.

Either brain-heart infusion (BHI) agar or broth (Difco) was used for growth, supplemented with 10 μg/ml of haemin chloride, 10 μg/ml of L-histidine and 2 μg/ml of β-NAD^+ (sBHI) [30]. Plate cultures were incubated at 36°C in 5% CO_2; liquid cultures were incubated at 37°C in air and shaken at 150 rpm.

Table 8.1 Description of multiple-resistant Spanish *Haemophilus influenzae* strains

H. influenzae strain[a]	Isolation year	Capsule type	Spanish geographic areas	Resistance	Source
R1661	1984	b	Northeast	Ap-Cm-Km-Tp	This study
R1664	1985	u	North	Ap-Cm-Km-Su-Te-Tp	This study
R1680	1985	u	Central	Ap-Cm-St-Su-Te-Tp	This study
R1696	1983	b	North	Ap-Cm-Km-St-Su-Te-Tp	This study
R1707	1984	b	North	Cm-Te-Tp	This study
R1708	1985	b	Northeast	Ap-Cm-Km-Te-Tp	This study
R1715	1982	b	Northeast	Ap-Cm-Km-Sm-Te-Tp	This study
R1716	1984	b	South	Ap-Cm-Km-Sm-Te-Tp	This study
R1728	1982	b	Northeast	Ap-Cm-Km-Sm-Te-Tp	This study
R1730	1983	b	Northeast	Ap-Cm-Te-Tp	This study
MAP	1972	u	NA	Em-Km-Sm-Sp	[8]
A8	1969	u	NA		[23]

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin hydrochloride; Su, sulfamethoxazole; Te, tetracycline; Tp, trimethoprim; u, nontypeable, NA, not applicable.

[a] Strains R1661, R1664 and R1680 were isolated from ear; R1696, R1708, R1716 and R 1730 from cerebrospinal fluid; R1707, R1715, and R1728 from blood.

Chemicals and biochemicals.

Restriction enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md) and used according to manufacturer's directions. Organic chemicals were purchased from Sigma (St. Louis).

Antimicrobial susceptibility studies.

Antimicrobial susceptibility testing was performed using the disk diffusion method with Mueller-Hinton agar supplemented with 1% haemin (Oxoid Laboratories, Basingstoke, UK) and 1% Iso-Vitalex (BBL, Cockeysville, Md). Trimethoprim and sulfamethoxazole disk susceptibility tests were performed on Iso-Sensitest agar (Oxoid Laboratories) supplemented with 5% lysed horse blood and 1% Iso-Vitalex. The strains were identified as susceptible or resistant according to National Committee for Clinical Laboratory Standards guidelines [26]. The minimal inhibitory concentrations (MICs) of ampicillin, chloramphenicol, tetracycline, streptomycin, kanamycin, trimethoprim and sulfamethoxazole were determined

by the agar dilution method using an inoculum of 10^4 colony-forming units (cfu) [5]. The strains were considered resistant if the MICs were equal or higher than the following concentrations: ampicillin, 4 μg/ml; chloramphenicol, 4 μg/ml; tetracycline, 8 μg/ml; kanamycin, 10 μg/ml, streptomycin, 10 μg/ml; trimethoprim, 5 μg/ml; and sulfamethoxazole, 200 μg/ml [6]. Trimethoprim and sulfamethoxazole resistance was confirmed by determination of the MIC on chemically defined medium [8] supplemented with haemin chloride and β-NAD, each at 10 μg/ml.

Enzymatic studies.

Rapid tests for β-lactamase and chloramphenicol acetyltransferase (CAT) were performed as previously described [2, 27]. Aminoglycoside phosphotransferase (APH) activity in bacterial cell sonicate supernatants was assayed by the method of Davies [10]. Strains were grown overnight in sBHI broth and pelleted by centrifugation at 15,000 g for 15 min at 4°C. Cells were resuspended in 1:10 volume of 20mM Tris Cl, 10 mM magnesium acetate, 25 mM NH_4Cl, and 0.6 mM 2-mercaptoethanol, pH 7.8, and sonified for 2 min at 50 W in 30-sec bursts with a Heat Systems (Plainview, NY) model W370 sonifier. After centrifugation at 20,000 g for 10 min at 4°C, the cell-free supernatant was used as a source of APH activity.The sonicate supernatant was assayed for APH activity after 5,10,15,30, and 60 min of incubation at 37°C using 25 mM kanamycin (final concentration) as the substrate and [^{32}P]ATP 0.12 μM. The amount of kanamycin-dependent product phosphorylated with time was plotted and the initial velocity calculated. Protein content of the cell extract was determined by a modification of the method of Lowry et al. as described [28]: APH specific activity was calculated from the initial velocity and protein content. Characterization of β-lactamases was carried out by analytic isoelectric focusing [36].

Isolation of plasmid DNA.

Cleared lysates of bacterial strains were prepared and subjected to agarose gel electrophoresis as described by Meyers et al. [25]. Plasmids were also sought using the procedure of Kado and Liu [16]. The size of the plasmids was determined using *Escherichia coli* plasmids as molecular weight standards [25]. Purification of plasmid DNA was carried out in CsCl-ethidium gradients by the large-scale method of Portnoy et al. [29]. The plasmids were banded twice in CsCl-ethidium bromide before analysis.

Probe preparation and hybridization methods.

Three different DNA probes were used. Plasmid pDM2 [21] containing an *H. parainfluenzae* β-lactamase was digested by *Bgl*I and *Bam*HI and electrophoresed in 1% agarose. A

tamase. Plasmid pGH54 encoding APH (3')1 [40] was digested with *Ava*I and electrophoresed in agarose. A 270-bp intragenic fragment was eluted, nicktranslated, and used as a probe for that gene. Plasmid pJRA1707, isolated as part of this study (Table 8.2), was digested with *Sst*I-*Xma*I and the gene for CAT cloned into the *Sst*I-*Xma*I site of pUC18 [39]. In vitro deletions yielded a 1.2-kilobase (kb) fragment, which encoded CAT and conferred low-level (25 μg/ml) chloramphenicol resistance in the *E. coli* strain DH5α ([13]; unpublished data). This 1.2-kb fragment was used as a probe for CAT. Purified plasmid DNA isolated from 10 representative transconjugants by CsCl-ethidium bromide gradient centrifugation was digested with *Eco*RI or *Sac*I and electrophoresed in 0.7% agarose gels. The DNA was then transferred to Nytran (Schleicher and Schuell, Keene, NH) by the method of Southern [33] as modified for bidirectional blotting by Smith and Summers [32]. Each probe was hybridized separately as described [38]. The filters were placed at -70°C for 48 h in a film holder with an intensifying screen and Kodak XAR-Z X-ray film.

Mating procedures.

Strain MAP (Table 8.1) was used as a recipient for the conjugation experiments. All 10 wild *H. influenzae* strains were mated separately with strain MAP by the filter paper

Table 8.2 Molecular sizes of plasmids and conjugation frequency of resistant *Haemophilus influenzae* after selection on 5μg/ml of chloramphenicol and 20 μg/ml of erythromycin.

Plasmid[a]	Size (Da x 10^6)	Frequency of conjugation	Unselected markers (%)		
			Ap	Te	Su
pJRA1661	42	2×10^{-5}	100	np	np
pJRA1664	48	3×10^{-5}	94	95	np
pJRA1680	48	2×10^{-5}	100	95	95
pJRA1696	50	7×10^{-6}	100	100	100
pJRA1707	38	1×10^{-4}	np	100	np
pJRA1708	45	2×10^{-5}	100	np	np
pJRA1715	50	6×10^{-5}	100	95	np
pJRA1716	50	1×10^{-5}	100	98	np
pJRA1728	50	8×10^{-6}	100	100	np
pJRA1730	42	9×10^{-6}	100	100	np

Abbreviations: Ap, ampicillin; Te, tetracycline; Su, sulfamethoxazole; np, resistance determinant not present in donor. Trimethoprim resistance was not detected in any of the transconjugants.

[a] Plasmid numbers correspond to numbers of donor strains.

technique as previously described [30]. Transconjugants were selected on two media: sBHI containing 5 μg/ml of chloramphenicol and 20 μg/ml of erythromycin and defined media [5] supplemented with haemin and β-NAD containing 5 μg/ml of trimethoprim and 20 μg/ml of erythromycin. The conjugation frequency of each strain was calculated by dividing the number of transconjugants by the number of donor cells plated. The spontaneous mutation frequency to erythromycin resistance was determined for all 10 donor strains by plating 10^8 bacteria on sBHI agar plates containing 20 μg/ml of erythromycin. One hundred representative transconjugants of each mating were plated on sBHI agar containing 50 μg/ml of spectinomycin hydrochloride and incubated overnight. Only strains growing on spectinomycin hydrochloride were identified as transconjugants and tested for other antibiotic resistance determinants.

Transformation.

Strain A8 [23] was rendered competent by the method of Herriott et al. [14] as modified by Stuy [35] with the addition of 32% glycerol. Purified plasmid DNA derived from a transconjugant of strain R1696 isolated on chloramphenicol- and erythromycin-containing media was used for transformation; transformants were selected on sBHI containing chloramphenicol at 3 μg/ml. Transformation to trimethoprim resistance was performed with whole-cell DNA (prepared by the method of Hull et al. [15]) derived from transconjugants of strains R1680, R1707, R1715, and R1716, which had been selected on sBHI agar containing chloramphenicol or trimethoprim. Strain MAP [7], rendered competent by the method of Herriott et al. [14], was used as a recipient. Transformants were selected on defined medium [8] supplemented with haemin and β-NAD^+ containing 5 μg/ml of trimethoprim. About 0.1 μg of DNA was added to aliquots of competent strain MAP. The transformation frequency was calculated by dividing the observed number of transformants by the number of competent cells used.

8.4 RESULTS

Transfer of resistance by conjugation.

All 10 wild-type strains (Table 8.1) transferred multiple drug resistance phenotypes by conjugation. When selection was performed on media containing chloramphenicol, the conjugation frequency ranged from 10^{-4} to 10^{-6} transconjugants per donor cell. All of the transconjugants examined contained plasmids (Figure 8.1). Resistance to ampicillin, tetracycline, and sulfamethoxazole was cotransferred with frequencies of 95% or higher, suggesting linkage. Concomitant transfer of resistance to trimethoprim was not detected in any of the strains tested, suggesting that linkage did not exist (Table 8.2). Transconju-

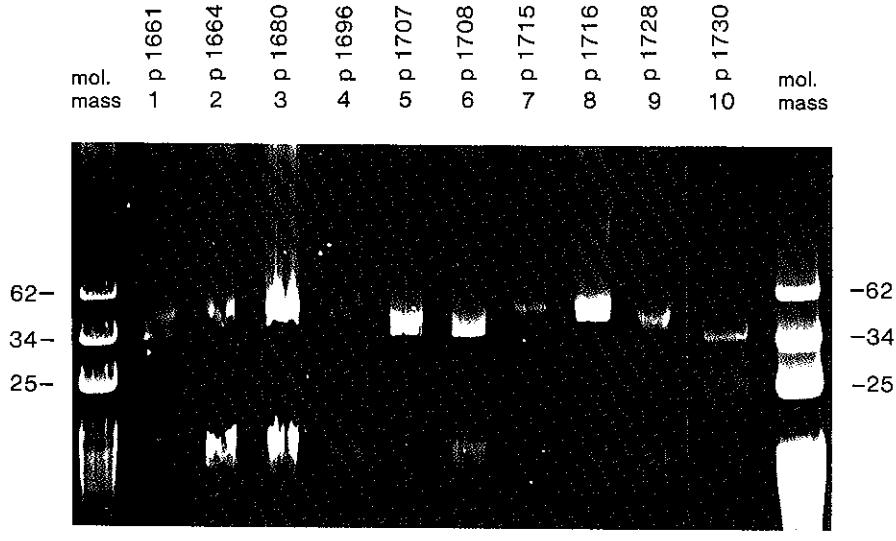

Figure 8.1 Cleared lysates of multiple-resistant *Haemophilus influenzae* transconjugants were electrophoresed in 0.7% agarose and stained with ethidium bromide. Transconjugants were derived from matings with strains designated as per plasmids. Molecular weight standards were derived from *Escherichia coli* [10].

gants could not be tested for resistance to streptomycin and kanamycin as the recipient strain MAP was resistant to these antibiotics. All of the transconjugants produced CAT, and all except those obtained from strain R1707 produced TEM-1 β-lactamase (data not shown) as determined by isoelectric focusing [36]. Transconjugants selected on chloramphenicol and erythromycin were examined for kanamycin phosphotransferase activity. Specific activity (expressed as μmol Pi/min per mg protein) ranged from 6.4×10^{-5} to 3.7×10^{-4}. *E. coli* PS 1328-24 harboring pGH54 [40], whose activity was 1.7×10^{-3}, was used as a positive control. The *H. influenzae* recipient strain MAP, which is thought to contain a ribosomal mutation conferring kanamycin resistance, had barely detectable phosphotransferase activity: the specific activity was 8-50 times less than that of the plasmid-containing transconjugants. The mutation frequency for resistance to 20 μg/ml erythromycin was undetectable ($< 10^{-9}$) for all 10 donor strains.

After filter mating and selection on media containing trimethoprim and erythromycin, putative transconjugants were found at a frequency of 10^{-5}-10^{-6}. The frequency of mutation of strain MAP to trimethoprim resistance was undetectable ($< 10^{-9}$). Putative trimethoprim-resistant transconjugants were susceptible to chloramphenicol, ampicillin, and tetracycline. These strains did not contain detectable plasmids when analyzed by the techniques of Meyers et al. [25] or of Kado and Liu [16]. The transconjugants yielded plasmids by both methods (Table 8.2).

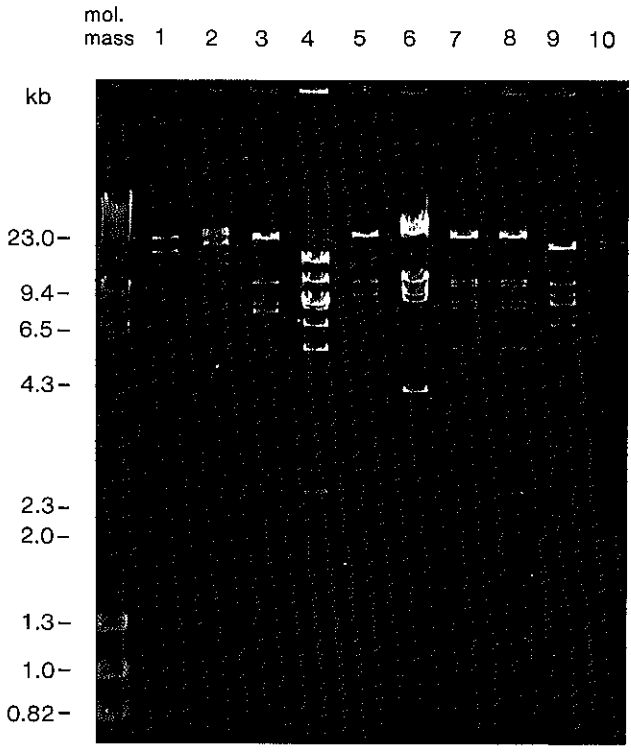

Figure 8.2 Purified plasmids from strain R1661 (lane 1), R1664 (lane 2), R1680 (lane 3), R1696 (lane 4), R1707 (lane 5), R1708 (lane 6), R1715 (lane 7), R1716 (lane 8), R1728 (lane 9), and R1730 (lane 10) were digested to completion with *Eco*RI, electrophoresed in 0.7% agarose, and visualized with ethidium bromide.

Plasmid DNA.

Lysis and electrophoresis of the 10 wild-type strains by the two methods used [16,25] did not consistently reveal plasmid DNA. Faint fluorescent bands could be seen in some lysates of wild-type strains. However, all 10 transconjugants selected on chloramphenicol-containing media harbored plasmids whose mass ranged from 38 to 50 MDa (Figure 8.1). These plasmids were detected with both methods of lysis. As noted, representative transconjugants from individual matings selected on trimethoprim did not contain detectable plasmid DNA when examined by the methods of Meyers et al. [25] or Kado and Liu [16]. In contrast, plasmid DNA was easily detected after lysis and electrophoresis of transconjugants selected on chloramphenicol.

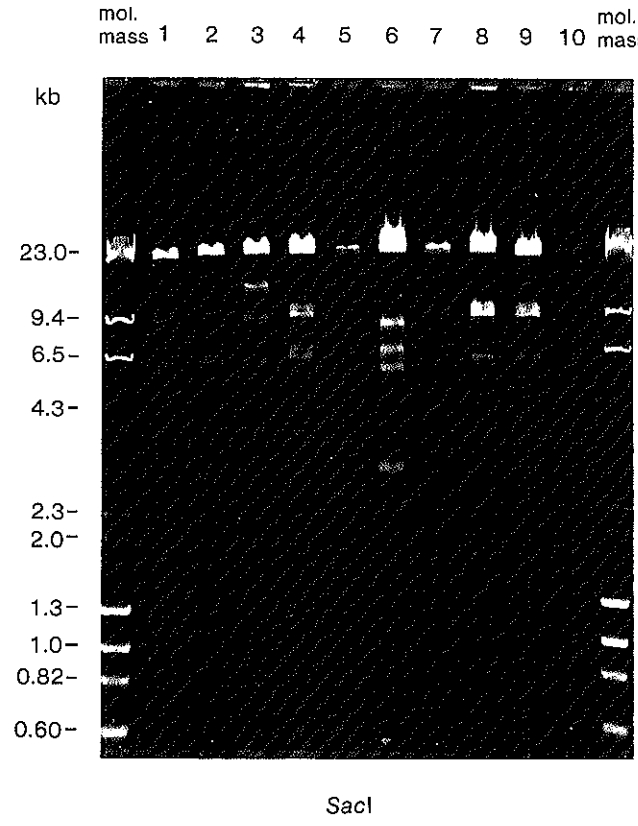

Figure 8.3 Purified plasmids processed identically to those in Figure 8.2, except digestion was performed with *Sac*I.

Transformation with chromosomal and plasmid DNA.

The frequency of transformation to trimethoprim resistance was 10^{-5} with DNA isolated from putative transconjugants selected on trimethoprim and was undetectable ($< 10^{-9}$) with DNA from transconjugants selected on chloramphenicol. To confirm that multiple resistance to other than trimethoprim was associated with plasmid DNA, we rendered strain A8 competent and transformed it with the 50-MDa plasmid isolated from a transconjugant of strain R1696. Transformation was detectable at a frequency of 2×10^{-9}; transformants were resistant to ampicillin, chloramphenicol, tetracycline, streptomycin, kanamycin and sulfamethoxazole. The transformant had β-lactamase and CAT activity and harbored plasmids with a mass identical to pJRA1696; all individual transformants were trimethoprim-susceptible.

Restriction endonuclease digestion.

Restriction endonuclease analysis revealed similarities among the plasmids isolated from strains from different geographic regions. Some degree of diversity was also found, as expected, by the different combinations of multiple resistance. More differences were seen after digestion with *Eco*RI than with *Sac*I (Figure 8.2 and 8.3). *Eco*RI and *Sac*I restriction fragments from R1715 and R1716 appeared identical even though the strains were isolated in two different regions; no other common patterns were found with *Eco*RI (Figure 8.2). *Sac*I restriction fragments appeared to be similar, if not identical, in plasmids isolated from transconjugants derived from matings with strains R1661, R1664, R1696, R1715, R1716 and R1728 (Figure 8.3); however, these varied in capsulation status and geographic site of isolation. Two additional individual restriction patterns, represented by strains R1680 and R1730, respectively, were also found.

DNA-DNA hybridization.

To determine the genetic relatedness of the plasmids from different geographic regions, we used three antimicrobial resistance gene probes to examine R-factors. *Eco*RI-digested plasmids (Figure 8.2) were hybridized with a ^{32}P-labelled 900-bp DNA fragment that codes for the TEM β-lactamase. Three different patterns of homology were seen (Figure 8.4). Plasmid pJRA1730 isolated in northeast Spain (Catalan region) had a hybridization pattern distinct from plasmids pJRA1728, pJRA1716, pJRA1715, pJRA1696 and pJRA1680 isolated in different regions of Spain (Table 8.1). As expected, plasmid pJC1707, isolated from an ampicillin-susceptible strain, showed no homology. Ampicillin-resistant plasmids pJRA1661, pJRA1664 and pJRA1708 showed weak homology. *Sac*I-digested plasmids were hybridized with a ^{32}P-labelled 270-bp DNA fragment from within the open reading frame of the APH(3')I gene. Only one homology pattern was seen (Figure 8.5). Kanamycin-susceptible plasmids pJRA1730 and pJRA1708 showed no homology. Plasmid

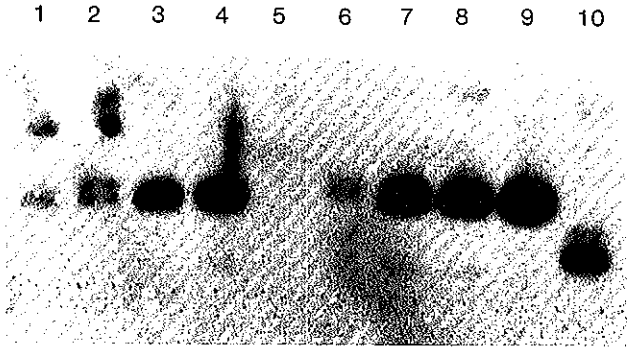

Figure 8.4 Southern analysis of *Eco*RI restricted plasmids using β-lactamase probe (see Materials and methods). Purified plasmids were derived from strains R1661 (lane 1), R1664 (lane 2), R1680 (lane 3), R1696 (lane 4), R1707 (lane 5), R1708 (lane 6), R1715 (lane 7), R1716 (lane 8), R1728 (lane 9), and R1730 (lane 10).

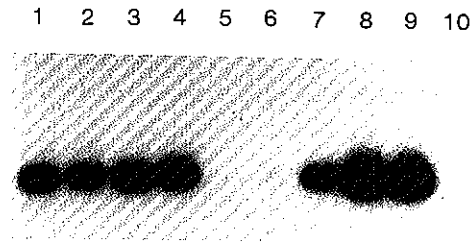

Figure 8.5 Southern analysis of *Sac*I restricted plasmids using the probe for aminoglycoside-phosphotransferase(3')I. Strains by lane are: lane 1, R1661; lane 2, R1664; lane 3, R1680, lane 4, R1696; lane 5, R1707; lane 6, R1708; lane 7, R1715; lane 8, R1716, lane 9, R1728; and lane 10, R1730.

pJRA1708 (lane 6) showed weak homology, although the strain was kanamycin-resistant and had kanamycin phosphotransferase activity.

Probing *Eco*RI-digested plasmids with the 1.2-kb fragment coding for CAT yielded a uniform pattern of hybridization. All plasmids contained a single homologous fragment (Figure 8.6, top). The signal was less intense with plasmids pJRA1661, pJRA1696, pJRA1708, pJRA1715 and pJRA1730. However, probing the *Sac*I digest revealed three different hybridization patterns (Figure 8.6, bottom). The hybridization pattern of the probe with the plasmid from which it was derived (Figure 8.6, bottom, lane 5) was also found with plasmids pJRA1664, pJRA1708 and pJRA1728 (Figure 8.6, bottom, lanes 2, 6 and 10). The most common hybridization pattern was seen with plasmids pJRA1661,

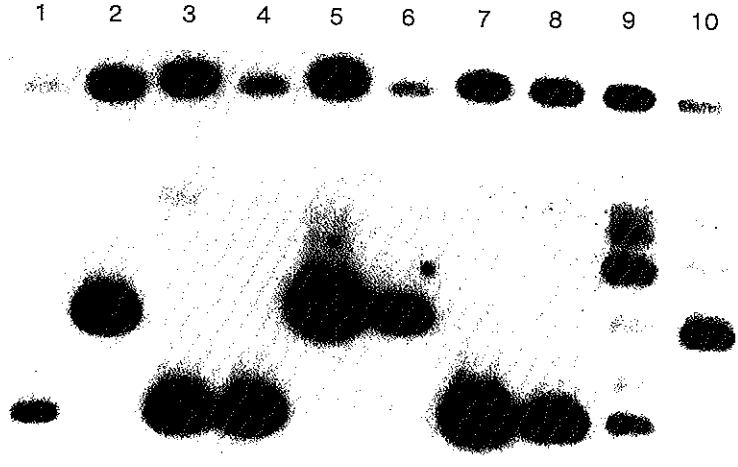

Figure 8.6 Plasmids purified from transconjugants of strains R1661 (lane 1), R1664 (lane 2), R1680 (lane 3), R1696 (lane 4), R1707 (lane 5), R1708 (lane 6), R1715 (lane 7), R1716 (lane 8), R1728 (lane 9), and R1730 (lane 10) were digested with *Eco*RI (top panel) or *Sac*I (bottom), electrophoresed in 0.7% agarose, and transferred to Nytran filters. A probe for chloramphenicol acetyltransferase was used.

pJRA1680, pJRA1696 pJRA1715 and pJRA1716 (Figure 8.6, lanes 1, 3, 4, 7 and 8). Plasmid pJRA1728 had a unique hybridization pattern (Figure 8.6, bottom, lane 9). Table 8.3 summarizes the relationship of the restriction analysis to the Southern analysis with the three probes with the 10 plasmids.

8.5 DISCUSSION

Resistance to multiple antibiotics is present in most clinically significant strains of *H. influenzae* isolated in Spain [3,4]. Although we could not consistently demonstrate plasmids in the wild-type strains, after filter-mating with an extensively passed *H. influenzae* strain (MAP) and selection for chloramphenicol resistance, plasmids of 38-50 MDa could be demonstrated in the transconjugants. This phenomenon has also been reported by others [24]. R-plasmids in these *H. influenzae* strains appeared to mediate resistance to ampicillin, chloramphenicol, tetracycline, sulfamethoxazole, and kanamycin but not trimethoprim. Trimethoprim resistance was present in all strains tested and could be mobilized with overnight filter mating but was not mediated by detectable plasmids. We previously have shown that the genetic basis of trimethoprim resistance in these and other strains is due to a chromosomal mutation; this has been found in a variety of *H. influenzae* [11]. Others have also noted the mobilization of *H. influenzae* chromosomal genes by cell-to-cell contact [1].

More restriction patterns were seen with *Eco*RI than with *Sac*I, as only plasmids pJRA1715 and pJRA1716 had the same patterns with *Eco*RI (Figure 8.2). These two plasmids, isolated from strains in northeast and southern Spain, respectively, appeared identical with all the genetic characteristics analyzed (Table 8.3); plasmid pJRA1696 isolated in northern Spain also showed the same pattern with *Sac*I and β-lactamase, APH (3')I, and CAT probes but the *Eco*RI restriction pattern was different. All three strains harbored plasmids with a mass of 50 MDa. Plasmid pJRA1730 appeared to be distinct from the rest. Plasmid pJRA1708 showed weak homology with the β-lactamase and APH(3')I probes, suggesting that more than one gene encoding each of these two enzymes may be present in Spain (Table 8.3). According to the restriction map published for pDM2 [21], the 900-bp *Bgl*I-*Bam*HI fragment is the structural gene for β-lactamase. We have found that this fragment encodes for β-lactamase in *E. coli* when cloned into a vector derived from pUC19 (unpublished data). This gene appears to be a partial or complete copy of the transposon Tn2, as both have three *Hin*cII sites (unpublished data). However, a portion of Tn2 is not present in the *bla* gene of pDM2 as Tn2 does not have a *Bgl*I site. Using the β-lactamase gene probe from pDM2 we found three patterns of hybridization (Figure 8.3).

Most kanamycin-resistant strains contained plasmids that hybridized with the internal

Table 8.3 Genetic relatedness of 10 plasmids isolated from multiple-resistant *Haemophilus influenzae*

	Restriction pattern		DNA hybridization pattern		
Plasmid	*Eco*RI	*Sac*I	βLac	APH(3')I	CAT
pJRA1661	A	a	1	1	α
pJRA1664	B	a-like	1	1	γ
pJRA1680	C	b	2	1	β
pJRA1696	D	a-like	2	1	β
pJRA1707	E	c	NA	NA	α
pJRA1708	F	c	?	?	α
pJRA1715	G	a-like	2	1	β
pJRA1716	G	a-like	2	1	β
pJRA1728	H	a-like	2	1	α
pJRA1730	I	a-like	3	NA	β

Abbreviations: βLac, β-lactamase; APH(3')I, 3'-aminoglycoside-phosphotransferase I; CAT, chloramphenicol-acetyltransferase; NA, not applicable: strain was susceptible to kanamycin or ampicillin; ?, signal on Southern blot analysis too weak for reliable interpretation. Restriction or hybridization patterns were placed in arbitrary groupings so that plasmids could be compared and groupings analyzed.

probe for APH(3')I, an enzyme for which a structural gene is present in strains of the family of Enterobacteriaceae [40]. However, only one restriction enzyme was used (*Sac*I) because the probe contained *Eco*RI and *Pst*I sites. The presence of APH(3')I in these *H. influenzae* differs from the findings reported by Levy et al. [20]: resistance to kanamycin and other 2-deoxystreptamine antibiotics in US *H. influenzae* isolates was neither mediated by R-plasmids nor by drug inactivation. The strains described in our report may be similar to the *H. influenzae* strain isolated in France [9]. Although multiple-resistant *H. influenzae* appear to have emerged suddenly in Spain, the hybridization pattern seen with probes for antibiotic resistance genes indicates that there was no dissemination of a single R-plasmid. Instead, it appears that a resident *Haemophilus* core plasmid acquired the *bla* and *cat* genes from various other bacteria; kanamycin resistance seems due to APH(3')I in most of the strains. These strains are also unique in that R-plasmid-mediated and chromosomal resistance occur in the same strains.

8.6 REFERENCES

1 **Albritton W.L., Setlow J.K., Slaney L.** *Transfer of Haemophilus influenzae chromosomal genes by cell-to-cell contact.* J Bacteriol 1982;152:1066-1070.

2 **Azemun P., Stull T.L., Roberts M.C., Smith A.L.** *Rapid detection of chloramphenicol resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1981;20:168-170.

3 **Campos J., García-Tornel S., Sanfellu I.** *Susceptibility studies of multiply resistant Haemophilus influenzae isolated from pediatric patients and contacts.* Antimicrob Agents Chemother 1984;25:706-709.

4 **Campos J., García-Tornel S., Musser J.M., Selander R.K., Smith A.L.** *Molecular epidemiology of multiply resistant Haemophilus influenzae type b in day care centers.* J Infect Dis 1987;156:483-489.

5 **Campos J., García-Tornel S., Gairi Tahull J.M., Fábregues I.** *Multiply resistant Haemophilus influenzae type b causing meningitis: comparative clinical and laboratory study.* J Pediatr 1986;108:897-902.

6 **Campos J., García-Tornel S.** *Comparative susceptibility of ampicillin and chloramphenicol resistant Haemophilus influenzae to fifteen antibiotics.* J Antimicrob Chemother 1987;19:297-301.

7 **Catlin B.W., Bendler III J.W., Goodgal S.H.** *The type b capsulation locus of Haemophilus influenzae: map location and size.* J Gen Microbiol 1972;70:411-422.

8 **Catlin B.W.** *Nutritional profiles of Neisseria gonorrhoeae, Neisseria meningitidis, and Neisseria lactamica in chemically defined media and the use of growth requirements for gonococcal typing.* J Infect Dis 1973;128:178-194.

9 **Dang van A., Goldstein F., Acar J.F., Bouanchaud D.H.** *A transferable kanamycin resistance plasmid isolated from Haemophilus influenzae.* Ann Inst Pasteur Microbiol 1975;126A:397-399.

10 **Davies J.E.** *Aminoglycoside-aminocyclitol antibiotics and their modifying enzymes.* In: Lorian V, ed. Antibiotics in laboratory medicine. 2nd ed. Baltimore: Williams & Wilkins, 1986:790-809.

11 **de Groot R., Campos J., Moseley S.L., Smith A.L.** *Molecular cloning and mechanism of trimethoprim resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1988;32:477-484.

12 **Garvey R.J.P., McMullin G.P.** *Meningitis due to beta-lactamase-producing type b Haemophilus influenzae resistant to chloramphenicol.* Br Med J 1983;287:1183-1184.

13 **Hanahan D.** *Studies on the transformation of Escherichia coli with plasmids.* J Mol Biol 1983;166:557-580.

14 **Herriott R.M., Meyer E.M., Vogt M.** *Defined nongrowth media for stage II development of competence in Haemophilus influenzae.* J Bacteriol 1970;101:517-524.

15 **Hull R.A., Gill R.E., Hsu P., Minshew B.H., Falkow S.** *Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection Escherichia coli isolate.* Infect Immun 1981;33:933-938.

16 **Kado C.I., Liu S.T.** *Rapid procedure for detection and isolation of large and small plasmids.* J Bacteriol 1981;145:1365-1373.

17 **Kenny J.F., Isburg C.D., Michaels R.H.** *Meningitis due to Haemophilus influenzae type b resistant to both ampicillin and chloramphenicol.* Pediatrics 1980;66:14-16.

18 **Kilian M.** *Haemophilus.* In: Lennette E.H., Balows A., Hauser Jr. W.J., Shadomy H.J. eds. Manual of clinical microbiology. 4th ed. Washington, DC: American Society for Microbiology, 1985:387-393.

19 **Levy J., Burns J.L., Mendelman P.M., Wong K., Mack K., Smith A.L.** *Effect of tobramycin on protein synthesis in 2-deoxystreptamine aminoglycoside-resistant clinical isolates of Haemophilus influenzae.* Antimicrob Agents Chemother 1986;29:474-481.

20 **McCarthy D., Clayton N., Setlow J.K.** *A plasmid cloning vehicle for Haemophilus influenzae and Escherichia coli.* J Bacteriol 1982;151:1605-1607.

21 **Mendelman P.M., Doroshow C.A., Gandy S.L., Syriopoulou V.P., Welgen C.P., Smith A.L.** *Plasmid-mediated resistance in multiply resistant Haemophilus influenzae type b causing meningitis: molecular characterization in one strain and review of the literature.* J Infect Dis 1984;150:30-39.

22 **Meyers J.A., Sanchez D., Elwell L.P., Falkow S.** *Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid.* J Bacteriol 1976;127:1529-1537.

23 **Michalka J., Goodgal S.H.** *Genetic and physical map of the chromosome of Haemophilus influenzae.* J Mol Biol 1969;45:407-421.

24 **Murphey-Corb M., Nolan-Willard M., Daum R.S.** *Integration of plasmid DNA coding for β-lactamase production in the Haemophilus influenzae chromosome.* J Bacteriol 1984;160:815-817.

25 **National Committee for Clinical Laboratory Standards.** *Approved standard M2-A3. Performance standards for antimicrobial disk susceptibility tests.* Villanova,PA: National Committee for Clinical Laboratory Standards, 1984.

26 **O'Callaghan C.H., Morris A., Kirby S.M., Shingler A.M.** *Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate.* Antimicrob Agents Chemother 1972;1:283-288.

27 **Peterson G.L.** *A simplification of the protein assay method of Lowry et al. which is more generally applicable.* Anal Biochem 1977;83:346-356.

28 **Portnoy D.A., Moseley S.L., Falkow S.** *Characterization of plasmids and plasmid-associated determinants of Yersinia enterocolitica pathogenesis.* Infect Immun 1981;31:775-782.

29 **Roberts M.C., Swenson C.D., Owens L.M., Smith A.L.** *Characterization of chloramphenicol-resistant Haemophilus influenzae.* Antimicrob Agents Chemother 1980;18:610-615.

30 **Simasathien S., Duangmani C., Echeverria P.** *Haemophilus influenzae type b resistant to ampicillin and chloramphenicol in an orphanage in Thailand.* Lancet 1980;II:1214-1217.

31 **Smith G.E., Summers M.D.** *The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper.* Anal Biochem 1980;109:123-129.

32 **Southern E.M.** *Detection of specific sequences among DNA fragments separated by gel electrophoresis.* J Mol Biol 1975;98:503-517.

33 **Stuy J.H.** *Effect of glycerol on Haemophilus influenzae transfection.* J Bacteriol 1986;166:285-289.

34 **Stuy J.H.** *Plasmid transfer in Haemophilus influenzae.* J Bacteriol 1979;139:520-529.

35 **Sykes R.B., Matthew M.** *The β-lactamases of Gram-negative bacteria and their role in resistance to β-lactam antibiotics.* J. Antimicrob Chemother 1976;2:115-157.

36 **Uchiyama N., Greene G.R., Kitts D.B., Thrupp L.D.** *Meningitis due to H. influenzae type b resistant to ampicillin and chloramphenicol.* J Pediatr 1980;97:421-424.

37 **van Klingeren B., van Embden J.D.A., Dessens-Kroon M.** *Plasmid-mediated chloramphenicol resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1977;11:383-387.

38 **Wahl G.F., Stern M., Stark G.R.** *Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization by using dextran sulfate.* Proc Natl Acad Sci USA 1979;76:3683-3687.

39 **Yanisch-Perron C., Vieira J., Messing J.** *Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 and mp18 and pUC19 vectors.* Gene 1985;33:103-119.

40 **Young S.A., Tenover F.C., Gootz T.D., Gordon K.P., Plorde J.J.** *Development of two DNA probes for differentiating the structural genes of subclasses I and II of the aminoglycoside-modifying enzyme 3'-aminoglycoside phosphotransferase.* Antimicrob Agents Chemother 1985;27:739-744.

CHAPTER 9

SUMMARY AND CONCLUSIONS

CHAPTER 9

SUMMARY AND CONCLUSIONS

This thesis was undertaken in order to investigate selected mechanisms of antibiotic resistance in *Haemophilus influenzae* (*H. influenzae*). As outlined in **chapter 1**, *H. influenzae* is a Gram-negative micro-organism responsible for a large variety of invasive and non-invasive infections in children and adults. Morbidity and mortality caused by encapsulated *H. influenzae* remain considerable especially in children below the age of five years. The important contribution of unencapsulated *H. influenzae* in the pathogenesis of childhood otitis media and respiratory tract infections has been well documented. The role of unencapsulated strains as causative agents of neonatal septicaemia and Brazilian purpuric fever is only recently documented. The choice of an adequate antibiotic therapy of *H. influenzae* infections has become complicated due to a significant increase in the prevalence of resistance to commonly used antibiotics such as ampicillin. The increased prevalence of antibiotic resistance in *H. influenzae* is not an isolated phenomenon, but it forms part of the global problem of antibiotic resistance in bacteria.

Chapter 2 discusses the basis of antibiotic resistance in micro-organisms. The available data on the evolution of worldwide prevalence of antibiotic resistance in the last decade indicate, that an overall increase is seen in resistance to trimethoprim, ampicillin and gentamicin [4]. Nevertheless, large differences exist in the levels of antibiotic resistance between different parts of the world or different regions. These differences may also depend on the characteristics of the sample collection and laboratory techniques. The highest levels of resistance are reported from developing countries. A survey of the prevalence of antimicrobial resistance performed by the Dutch National Institute of Public Health and Environmental Protection in 1989 and 1990 indicates, that the level of resistance to ampicillin, trimethoprim and gentamicin in the Netherlands is lower, than in most other countries. Some preliminary data from this study were kindly provided by Dr. B. van Klingeren. The results obtained from MIC-determination of thousands of bacterial isolates indicate that resistance of *Enterobacteriaceae* to gentamicin is below 5%. Trimethoprim resistance in *E. coli* and *K. pneumoniae* appears to be stable at approximately 25%, which is substantially less, than the Tmp resistance rates reported from

several developing and developed countries (see chapter 3). Tmp resistance in *S. aureus* is below 3%. B-lactamase-mediated resistance to ampicillin in *E. coli* and *Proteus spp.* remains stable at approximately 30 respectively 12% after an initial increase in the 1970s. B-lactamase producing *H. influenzae* comprise approximately 7% of the total number of isolates.

One of the major factors involved in the increased prevalence of resistant hospital micro-organisms is the selection pressure exerted by the administration of antibiotics. The impact of veterinary use of antibiotics on the emergence of resistance in bacteria causing disease in man is still uncertain [7]. The potential effects of selection of antibiotic-resistant micro-organisms in veterinary medicine and the rapid spread to a human reservoir may be illustrated by recent data from Dr. H.Ph. Endtz (personal communication). Studies with *Campylobacter spp.* isolated from humans show, that the level of resistance to the quinolone enrofloxacin, which is exclusively used in domestic animals, has increased from almost 0 to 20% in the last three years. The presence of a mechanism for intensive exchange of antibiotic resistance genes between bacteria pathogenic for humans and animals, including *H. influenzae*, has been well documented. It has also become evident that infections caused by resistant micro-organisms are associated with a higher risk for morbidity and mortality.

One of the most striking characteristics of antibiotic resistance in bacteria is the enormous heterogeneity in the molecular regulation, the transfer mechanisms of resistance genes, and the biochemical mechanisms of resistance. Six major genetic mechanisms of resistance to antibiotics are described. Four of these, namely intrinsic resistance, chromosomal mutation to resistance, plasmid-mediated resistance and transposon-mediated resistance are commonly found in *H. influenzae* as described in chapters 4 until 8. Antibiotic resistance genes may be transferred from one micro-organism to another by means of conjugation, transformation, transduction and transposition. The experiments in chapters 5 and 8 delineate the importance of conjugation and transformation as tools for the study of antibiotic resistance genes in *H. influenzae.* Transposition of antibiotic resistance genes is also extensively characterized in *H. influenzae* (see chapter 4). Although many *H. influenzae* isolates contain bacteriophages, antibiotic resistance due to transduction has to our knowledge not been described. The biochemical mechanisms of antibiotic resistance in micro-organisms may be classified in seven groups (Table 2.2, chapter 2). The potential of *H. influenzae* strains to develop different biochemical mechanisms of resistance is remarkable, since six of these seven mechanisms have been detected (see chapter 4). The division of antimicrobial resistance into two major categories termed "positive function resistance" and "persistence" may also be applied to antibiotic resistance in *H. influenzae*. Examples of positive function resistance are the plasmid-mediated resistance mechanisms in *H. influenzae* to ampicillin, chloramphenicol, tetracycline and aminoglycosides, which are described in chapter 4. The control of dissemination of this type of resistance should be directed to the development of new

antibiotics, to a decrease in the use of antibiotics in the population at large and to an effective policy of prevention of dissemination of these plasmids in the hospital environment by appropriate isolation measures.
Examples of persistence as a second mechanism of resistance in *H. influenzae* include chromosomal mutations in penicillin-binding proteins, decreased permeability of *H. influenzae* for chloramphenicol and thymine auxotrophy (see chapter 3). The clinical isolates with these types of resistance are commonly cultured during antibiotic treatment of chronic respiratory infections as shown in chapter 5 of this thesis. Auxotroph studies indicated, that these strains are additionally altered in their requirement for different amino acids. It has been shown, that a removal of the antibiotic selection pressure in these isolates often leads to a reversal to the initial phenotype and/or genotype. Prevention of persistence may include the use of more than 1 antibiotic, the application of antibiotics with different mechanisms of action, and a more critical selection of antibiotics in the individual patient.

Chapter 3 summarizes the mode of action and the clinical use of trimethoprim or co-trimoxazole in the treatment of infections caused by Gram-positive and Gram-negative micro-organisms. The mechanisms of resistance to trimethoprim in micro-organisms are reviewed. The major mechanisms are plasmid- or transposon-encoded production of a trimethoprim-resistant dihydrofolate reductase (DHFR) or overproduction of DHFR. The heterogeneity between the different altered DHFRs in Gram-positive and Gram-negative micro-organisms is striking. The study of the molecular regulation and the biochemical characteristics of DHFRs has provided new insight into the evolution and spread of antibiotic resistance genes. The rapid emergence of trimethoprim resistance in Enterobacteriaceae, *Salmonella* and *Shigella* has become a major problem, especially in developing countries, in the management of infections caused by these micro-organisms. Trimethoprim is also an important antibiotic in the treatment of upper and lower respiratory tract infections caused by *H. influenzae*. This formed the rationale for the studies reported in chapters 5, 6 and 7 of this thesis on the genetic basis and the biochemical mechanisms of resistance to trimethoprim in *H. influenzae*.

Chapter 4 briefly discusses the risk factors, the diseases and the preferential choice of antibiotics in infections caused by encapsulated and unencapsulated *H. influenzae*. The problems with in vitro susceptibility testing of *H. influenzae* and the necessity of standardization of MIC testing are emphasized. Reliable MIC determinations are influenced by factors such as differences in the composition of growth media, the size of the bacterial inoculum, and the inhibition of certain antibiotics by constituents in media. This has particularly complicated the accurate detection of ampicillin-resistant non-β-lactamase producing *H. influenzae* and of trimethoprim-resistant isolates.
The epidemiology and the genetic and biochemical mechanisms of resistance to a large variety of antibiotics are discussed. These include resistance to clinically important

compounds such as ampicillin and various other β-lactam antibiotics, chloramphenicol, trimethoprim and resistance to multiple antibiotics. The major mechanism of resistance to ampicillin is the production of a TEM β-lactamase, which is located on a 3 x 10^6 dalton sequence of DNA, called transposon A (TnA). TnA is probably derived from plasmids in other Gram-negative micro-organisms. TEM β-lactamase is also the cause of ampicillin resistance in the multiple-resistant *H. influenzae* strains in chapter 8. Non β-lactamase producing ampicillin-resistant *H. influenzae* are increasingly reported. The mechanism of resistance in these strains is a mutation in the penicillin-binding proteins resulting in a decrease in affinity for different β-lactam antibiotics. Resistance to chloramphenicol is mainly caused by the plasmid-mediated production of chloramphenicol acetyltransferase as also shown in chapter 8. The molecular and biochemical mechanisms of trimethoprim resistance form the subject of the studies in chapters 5, 6 and 7.
The clinical implications of the increasing prevalence of antibiotic resistance in *H. influenzae* are discussed. In addition, the preferential choice of antibiotics in infections caused by unencapsulated and encapsulated *H. influenzae* is reviewed and summarized in Tables 4.1 and 4.2. The first choice agent in the treatment of susceptible *H. influenzae* remains ampicillin. Meningitis may alternatively be treated with several third-generation cephalosporins. Other effective antibiotics in the treatment of pneumonia, otitis media, sinusitis and chronic respiratory tract infections include amoxicillin-clavulanate, cefuroxime and trimethoprim-sulphamethoxazole.

Chapter 5 reports the molecular cloning and preliminary characterization of the mechanism of trimethoprim resistance in *H. influenzae*. Ten trimethoprim-resistant (Tmp^r) unencapsulated *H. influenzae* isolates were detected by agar dilution MICs on a thymidine-free medium. The MICs of these strains varied between 10 μg/ml to > 200 μg/ml. Susceptible strains had a MIC of < 0.5 μg/ml. Trimethoprim resistance could be transferred at high frequencies in both conjugation and transformation experiments. Although the transfer of resistance genes in the conjugation experiments suggests the presence of plasmids, we could not detect plasmids in wild-type Tmp^r strains and Tmp^r transconjugants. We therefore concluded, that trimethoprim resistance in *H. influenzae* is chromosomally encoded and that transfer of resistance genes in mating experiments may find place through a yet unknown cell-to-cell contact mechanism. Albritton et al. have previously published similar experiments supporting the presence of this mechanism [1]. The trimethoprim resistance gene from strain R1047 (MIC 40 μg/ml) was cloned into a cosmid vector and recombinant plasmids were transduced into *E.coli* HB101. Six recombinant cosmid clones resistant to both ampicillin and trimethoprim were selected on L agar plates. Transformation experiments with plasmid DNA from these clones yielded Tmp^r transformants at a frequency of 1 x 10^{-3}. A 12.9-kb *Eco*RI fragment of one of the plasmids was subcloned into pGEM3. This plasmid was called pRGS7 and still conferred trimethoprim resistance. A restriction enzyme map of pRGS7 was generated (Figure 5.1).

Additional transformation experiments with whole-cell restriction enzyme digested DNA from Tmp^r strains and with different restriction enzyme fragments from pRGS7 indicated the approximate location of the gene encoding trimethoprim resistance. A 500 basepair *Xba*I-*Pst*I intragenic DNA fragment was labeled with ^{32}P and used as a probe in both colony-hybridization and Southern blot experiments. On colony blots the probe hybridized to a variety of unencapsulated and encapsulated Tmp^r *H. influenzae* strains. However, the probe also hybridized with capsulated and unencapsulated trimethoprim-susceptible strains. Hybridization was not seen with DNA from Tmp^r *Escherichia coli* containing dihydrofolate reductase I, II and III genes or with Tmp^r *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Pseudomonas cepacea*. These data suggested that the genes encoding trimethoprim resistance in these micro-organisms are substantially different from the trimethoprim resistance gene in *H. influenzae*. This is not surprising considering the major differences between bacterial DHFRs in amino acid sequence and nucleotide sequence (see chapter 3). Southern blot analysis with restriction enzyme digested DNA from isogenic trimethoprim-susceptible and trimethoprim-resistant strains using the 500 basepair *Xba*I-*Pst*I probe showed, that the probe hybridized with fragments of different size in DNA restricted with *Dde*I and *Hin*fI. It was concluded from these data, that the acquisition of trimethoprim resistance involved a rearrangement or change in nucleotide sequence. The ^{32}P-labeled *Xba*I-*Pst*I probe was subsequently used in Southern blot experiments to answer the question if resistance to trimethoprim was indeed chromosomally encoded. Plasmid and chromosomal DNA from multiple-resistant *H. influenzae* isolates from Spain and from Tmp^r transconjugants of these strains (both extensively characterized in chapter 8 of this thesis) was hybridized with the *Xba*I-*Pst*I probe. The probe hybridized with the chromosomal DNA from all Tmp^r strains and showed no homology with plasmid DNA from trimethoprim-resistant transconjugants, thus supporting the notion, that trimethoprim resistance is chromosomally encoded.

We subsequently investigated the mechanism of resistance to trimethoprim in *H. influenzae*. Analysis of outer membrane protein profiles and trimethoprim uptake experiments (both not reported) showed no differences between trimethoprim-susceptible and -resistant *H. influenzae*. However, measurement of DHFR activity in the cell sonicate supernatant of trimethoprim resistance strains showed significantly greater activity in comparison with DHFR activity in trimethoprim-susceptible strains. From these data it was concluded, that trimethoprim resistance in *H. influenzae* was caused by an overproduction of DHFR. Preliminary investigation of the trimethoprim inhibition profiles in isogenic-susceptible and -resistant strains showed a similar 50% trimethoprim inhibition constant. However, these experiments on crude enzyme extracts did not exclude the possibility, that additional structural or functional differences existed between the DHFRs in trimetho prim-susceptible and trimethoprim-resistant strains. We therefore decided to purify and characterize the DHFRs from these strains in order to answer this question.

Chapter 6 describes the partial purification and characterization of DHFR from a trimethoprim-susceptible (Tmp^s strain MAP) and two trimethoprim-resistant (Tmp^r strains MAP/47 and MAP/42) of *H. influenzae*. The enzymes were purified between 650- and 3000-fold by a combination of ammonium-sulphate fractionation, size separation on a Sephadex G-75 column and affinity chromatography on a Matrix Gel Green A column. Total enzyme activity of the final electrophoretically pure preparations from MAP/47 and MAP/42 was 6-10-fold higher than those derived from MAP, whereas the specific activity in units/mg protein was approximately similar at the end of the purification procedure. This confirmed our previous conclusion in chapter 5 that trimethoprim resistance in *H. influenzae* is caused by overproduction of chromosomally encoded DHFR. Estimation of the molecular mass of the three DHFRs by SDS/PAGE indicated a protein with a relative mobility equivalent to 18.4 kDa. Michaelis-Menten kinetic analyses with highly purified DHFR fractions showed, that the Km for dihydrofolate (7, 9 and 5 μM) and NADPH (2, 5 and 6 μM) was also similar in all three enzymes. However, significant differences were found between the trimethoprim IC_{50} (the concentration necessary for 50% inhibition of DHFR activity) from the Tmp^s strain MAP (0.001 μM) and the Tmp IC_{50} values from MAP/47 and MAP/42 (0.1 μM and 0.3 μM respectively). The methotrexate IC_{50} of MAP/42 DHFR was also higher (0.06 μM) in comparison with the enzymes from MAP (0.008 μM) and MAP/47 (0.007 μM). These results suggested that trimethoprim resistance in *H. influenzae* was not exclusively caused by overproduction of DHFR, but also by structural alterations in the DHFRs from Tmp^r strains. Additional support for these data was derived from isoelectric focusing experiments with the three DHFR fractions. DHFR from MAP/47 had a different isoelectric point (pI 7.6) compared with the enzymes from MAP and MAP/47 (pI 7.3). In addition, peptide mapping after digestion with trypsin revealed one major peptide fragment (7.9 kDa) in the DHFR of MAP and MAP/47 and three major tryptic fragments (7.9, 9.6 and 12.5 kDa) in DHFR from MAP/42. One may conclude from these data, that structural and functional differences exist between DHFRs from each of the three Tmp^s and Tmp^r strains. We hypothesized, that the genetic basis of trimethoprim resistance in *H. influenzae* was similar to the one described in regulatory mutants of DHFR in *Escherichia coli* K12 [5]. Smith & Calvo studied the molecular basis of trimethoprim resistance in *Escherichia coli* mutants selected by in vitro mutagenesis experiments [6]. They revealed, that overproduction of DHFR in these isolates was caused by a mutation in the promoter, whereas the decreased affinity for trimethoprim could be explained by a mutation in the "fol" gene. We decided to investigate the genetic basis of trimethoprim resistance in *H. influenzae* by subcloning, sequencing and determination of the protein product and the promoter regions of the gene encoding trimethoprim resistance in strain R1047 [cloned into pGEM3 (pRGS7) see chapter 5].

Chapter 7 describes the subcloning and nucleotide sequence of a 1144 basepair (bp) DNA fragment from *H. influenzae* strain R1047, that confers resistance to trimethoprim.

The DNA sequence has two open reading frames (ORFs) which contain respectively 480 and 561 bp.

The first ORF specifies a protein with a predicted molecular mass of 17760 Da. The deduced amino acid sequence has 51.3% homology in an overlap of 158 amino acids with DHFR from *E. coli* K12. Homology with other prokaryotic Tmp^s and Tmp^r DHFRs varied between 34.9% and 49.1%. The molecular mass of this DHFR is similar to the calculated 18400 Da. of purified DHFR as reported in chapter 6. We questioned if the overproduction of altered DHFR in *H. influenzae* strain R1047 could be caused by mutational changes in the structural "fol" gene and the promoter region. The extensive structural dissimilarities between *H. influenzae* R1047 DHFR and other DHFRs make it difficult to assess, if changes in binding site(s) reflect functional changes in DHFR or if they are a consequence of normal variability between DHFRs from different micro-organisms. Therefore definitive conclusions concerning the nature of these changes will need to wait until sequence data are available from the Tmp^s strain R906 and the highly Tmp-resistant *H. influenzae* isolate R1042. However, comparison of the structural "fol" gene and the 5' upstream regulatory sequences from R1047 DHFR with DHFR "fol" and promoter sequences from several Gram-positive and Gram-negative micro-organisms shows some interesting points. The amino acid residues which are part of the hydrophobic binding pocket of DHFR or which have been implicated in the interaction of DHFR with NADPH or folate antagonists are - as expected - highly conserved in *H. influenzae* R1047 DHFR (Figure 7.3, chapter 7). It has been shown, that the substitution of a single amino acid residue in one of the regions mentioned above may lead to major changes in inhibitor binding and kinetic properties of DHFR. Such a change may have occurred in the highly conserved glutamate residue at position 121, resulting in a replacement by aspartate.

Comparison between the promoter region of the R1047 "fol" gene and several other DHFR promoter sequences indicates, that the -10 sequence is completely identical to the -10 sequence from *E. coli* K12, whereas the -35 sequence is identical in 5 of 6 nucleotides to the -35 region of *E. coli* 1810, which overproduces DHFR several hundredfold. The presence of a T in position -34 of the -35 sequence may also be important, since it has been reported, that a C to T conversion at this position results in a 12- to 15-fold increased synthesis of "fol" mRNA in mutant *E. coli* strains. We speculate that changes in the -35 and/or -10 region similar to those seen in *E. coli* mutant strains may be responsible for the overproduction of DHFR in *H. influenzae* R1047. We intend to study the gene regulation of R1047 "fol" in further detail by oligonucleotide site-directed mutagenesis. In future experiments we aim to replace the A at position -35 in a T, which will result in a TTG sequence. It has previously been shown, that this sequence is characteristic for strong Gram-negative promoters and we therefore expect, that the conversion indicated above will result in a further significant increase in DHFR production. In addition, study of

the ribosome binding site (RBS) from Tmp^r and Tmp^s "fol" genes will elucidate if the position of the RBS in relation to the ATG start codon and the amount of homology with the 3' end of 16S rRNA may also contribute towards overproduction of DHFR in Tmp^r *H. influenzae*. We therefore intend to sequence the DHFR gene and the regulatory sequences from Tmp^r strain R1042 and Tmp^s strain R906. The genome of interest will be amplified by means of the polymerase chain reaction (PCR) using primers from 5' upstream and 3' downstream sequences of the "fol" gene from R1047. *Eco*RI sites will be incorporated into the primers to be able to clone the PCR product into a commercial vector.

The second ORF 5' of the DHFR gene on the complementary strand is transcribed in the opposite direction and contains 561 nucleotides. Analysis of the ORF indicates 62.9% identity with the first 187 amino acids of the N-terminal sequence of the *E. coli* proBA region. The region upstream from the 5'-terminus of the proB gene contains a putative ribosome binding site and -10 and -35 promoter sequences (Figure 8.2b). The proB gene encodes γ-glutamyl kinase, which activates glutamate to γ-glutamyl phosphate. The second step in this pathway from glutamate to proline is the conversion of γ-glutamyl phosphate into glutamate γ-semialdehyde by the enzyme glutamate semialdehyde dehydrogenase. This enzyme is encoded by the proA gene, which forms part of the proBA operon. Proline has an important role in bacterial protein synthesis and serves as a carbon and nitrogen source. It is interesting, that the proBA map position in *E. coli* (at 6 min) is far distanced from the DHFR "fol A" gene (at 1 min), whereas these two genes are clustered in the *H. influenzae* genome. It is yet unclear if this clustering has any functional significance.

The subject of **chapter 8** is the study of the genetic relatedness of antibiotic resistance determinants in multiple-resistant *Haemophilus influenzae*. *H. influenzae* isolates, which are resistant to ampicillin, chloramphenicol, and tetracycline with or without resistance to trimethoprim, kanamycin, sulfamethoxazole and streptomycin have become a major problem in Spain and some other European countries [2, 3]. Ten multiple-resistant *H. influenzae* isolates were studied in order to answer two questions: 1) are multiple-resistant *H. influenzae* from different geographic regions due to one single plasmid clone or from polyclonal origin and 2) are the antibiotic resistance genes identical or dissimilar. Antibiotic resistance to ampicillin, chloramphenicol, tetracycline, kanamycin, streptomycin and sulfamethoxazole was encoded by conjugative plasmids with molecular masses of 38-50 MDa. In contrast, resistance to trimethoprim, which could be transferred in both conjugation and transformation experiments, was not plasmid-encoded but chromosomally mediated (see also chapter 5). Enzymatic studies to reveal the mechanism of resistance to ampicillin, chloramphenicol and kanamycin in transconjugant strains included rapid tests for β-lactamases and chloramphenicol acetyltransferase (CAT) and analysis of aminoglycoside phosphotransferase in cell sonicate supernatants. All of the transcon-

jugant strains produced CAT and all except one produced TEM-1 β-lactamase. Transconjugants selected on chloramphenicol and erythromycin all had 8-50 times more kanamycin phosphotransferase activity than the kanamycin susceptible recipient strain MAP. Restriction endonuclease digestion of the isolated plasmids revealed similarities, but also some degree of heterogeneity depending on the restriction enzyme used. Southern blot analysis of these plasmids digested with different restriction enzymes and hybridized with antimicrobial resistance gene probes, for TEM-1 β-lactamase, chloramphenicol acetyltransferase (CAT) and kanamycin phosphotransferase indicated that 10 plasmids had different combinations of hybridization patterns. These experiments further supported the notion, that multiple antibiotic resistance phenotypes in *H. influenzae* do not arise from acquisition of a single R-plasmid. It may be concluded that both plasmid resistance (to ampicillin, chloramphenicol and kanamycin) and chromosomal resistance (to trimethoprim) evolved independently from several strains.

9.1 REFERENCES

1 **Albritton W.L., Setlow J.K., Slaney L.** *Transfer of Haemophilus influenzae chromosomal genes by cell-to-cell contact.* J Bacteriol 1982;152:1066-1070.

2 **Campos J., García-Tornel S., Galri Tahull J.M.** *Invasive infections caused by multiply resistant Haemophilus influenzae type b.* J Pediatr 1984;104:162-163.

3 **Campos J., García S., Galri Tahull J.M., Fábregues I.** *Multiply resistant Haemophilus influenzae type b causing meningitis:comparative clinical and laboratory study.* J Pediatr 1986;108:897-902.

4 **O'Brien T.F. and members of Task Force 2** *Resistance of Bacteria to antimicrobial agents: report of task force 2.* Rev Infect Dis 1987;9:S244-S260.

5 **Sheldon R.** *Altered dihydrofolate reductase in fol regulatory mutants of Escherichia coli K12.* Molec Gen Genet 1977;151:215-219.

6 **Smith D.R., Calvo J.M.** *Nucleotide sequence of dihydrofolate reductase genes from trimethoprim-resistant mutants of Escherichia coli.* Mol Gen Genet 1982;187:72-78.

7 **Workshop Rijksinstituut voor Volksgezondheid en Milieuhygiëne.** *Veterinair antibioticagebruik en volksgezondheid.* Bilthoven 22 maart 1990.

SAMENVATTING EN CONCLUSIES

In dit proefschrift wordt verslag gedaan van een onderzoek naar mechanismen die een rol spelen bij het ontstaan van resistentie tegen antibiotica in *Haemophilus influenzae* (*H. influenzae*). Zoals in **hoofdstuk 1** geschetst, is *H. influenzae* een Gram-negatief micro-organisme dat verantwoordelijk is voor een grote verscheidenheid aan infecties. Ziekte- en sterfgevallen als gevolg van gekapselde *H. influenzae* komen vooral voor bij kinderen onder de vijf jaar. Niet-gekapselde *H. influenzae* zijn een belangrijke oorzaak van middenoorontsteking en infecties van de onderste en bovenste luchtwegen. Sinds kort is bekend dat niet-gekapselde stammen tevens sepsis bij pasgeborenen en "Brazilian purpuric fever" kunnen veroorzaken. Het instellen van een adequate behandeling tegen *H. influenzae* wordt echter bemoeilijkt door de toenemende resistentie tegen veel gebruikte antibiotica zoals ampicilline. Deze resistentie is niet een op zichzelf staand verschijnsel, doch vormt een onderdeel van het over de gehele wereld bestaande probleem van bacteriële resistentie tegen antibiotica.

Hoofdstuk 2 is gewijd aan de bespreking van resistentie tegen antibiotica in micro-organismen. De beschikbare gegevens wijzen op een toeneming van de resistentie tegen trimethoprim, ampicilline en gentamicine [4]. Er bestaan echter grote verschillen tussen verschillende werelddelen, en ook tussen diverse regio's in de mate van resistentie tegen antibiotica. Deze verschillen kunnen mede veroorzaakt worden door variaties in de selectiemethoden bij het afnemen van monsters en door het gebruik van verschillende laboratoriumtechnieken. Het hoogste resistentie-niveau wordt gemeld uit ontwikkelingslanden. Uit een onderzoek van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne, uitgevoerd in 1989 en 1990, blijkt dat in Nederland het niveau van resistentie tegen ampicilline, trimethoprim en gentamicine lager is dan in vele andere landen. Dr. B. van Klingeren was bereid enige voorlopige gegevens uit dit onderzoek voor publicatie hier beschikbaar te stellen. Gevoeligheidsbepalingen van duizenden bacteriën tonen, dat de resistentie tegen gentamicine in *Enterobacteriaceae* minder dan 5% bedraagt. Resistentie tegen trimethoprim (Tmp) in *E. coli* en *K. pneumoniae* lijkt stabiel te zijn (ca. 25%). Dit is aanzienlijk lager dan gerapporteerd wordt (speciaal met betrekking tot Tmp-resistentie) uit verscheidene andere landen, zowel in de Derde als in de Westerse Wereld (zie hoofdstuk 3). Tmp-resistentie in *S. aureus* blijkt onder de 3% te liggen. De resistentie tegen ampicilline in *E. coli* en *Proteus spp.*, die wordt veroorzaakt door β-lactamase, blijft stabiel rond de 30, respectievelijk 12% na aanvankelijk toegenomen te zijn in de jaren '70. Zeven procent van in Nederland geïsoleerde *H. influenzae*-stammen vormt β-lactamase.

Eén van de belangrijkste oorzaken van de toeneming van het aantal resistente micro-organismen in ziekenhuizen is selectie van resistente bacteriën tijdens het gebruik van antibiotica. De causale relatie tussen het veterinaire gebruik van antibiotica en het ontstaan van resistentie in bepaalde micro-organismen die ziekte bij mensen veroorzaken, is nog niet volledig bewezen [6]. De mogelijke gevolgen van selectie van resistente micro-organismen in de diergeneeskunde en de snelle verspreiding van deze bacteriën naar de mens worden echter geïllustreerd door gegevens van Dr. H.Ph. Endtz (persoonlijke mededeling). Studies met *Campylobacter spp.*, geïsoleerd bij mensen, tonen aan dat het niveau van resistentie tegen het quinolone enrofloxacin, dat uitsluitend bij vee gebruikt wordt, is toegenomen van 0 tot 20% gedurende de afgelopen vijf jaar. De uitwisseling van antibiotische resistentie-genen tussen micro-organismen voorkomend bij mens en dier is ook voor andere bacteriën beschreven. Infecties, veroorzaakt door resistente micro-organismen brengen daarnaast een hoger risico voor ziekte en overlijden met zich mee.
Opvallend bij de resistentie tegen antibiotica in bacteriën is de zeer grote heterogeniteit in de moleculaire regulatie, in de wijze van overdracht van resistentie-genen en in de verschillende mechanismen van resistentie. De zes bekende genetische mechanismen van resistentie tegen antibiotica worden hieronder genoemd. Vier van deze, nl. intrinsieke resistentie, chromosomale mutaties, door plasmiden en door transposon gecodeerde resistentie, treft men vaak aan bij *H. influenzae*, zoals beschreven in de hoofdstukken 4 t/m 8. Antibiotische resistentie-genen kunnen van het ene micro-organisme naar het andere overgebracht worden door middel van conjugatie, transformatie, transductie en transpositie. Dat conjugatie en transformatie belangrijk zijn voor de studie van antibiotische resistentie-genen in *H. influenzae*; wordt aangetoond aan de hand van experimenten die beschreven zijn in de hoofdstukken 5 en 8. Transpositie van antibiotische resistentie-genen in *H. influenzae* wordt ook uitgebreid beschreven (zie hoofdstuk 4). Resistentie tegen antibiotica als gevolg van transductie is, voor zover ons bekend, ook door anderen niet beschreven, hoewel vele *H. influenzae*-isolaten bacteriofagen bevatten.

De biochemische mechanismen van antibiotische resistentie in micro-organismen kunnen in zeven rubrieken ondergebracht worden (tabel 2.2, hoofdstuk 2). De verscheidenheid aan antibiotische resistentie-mechanismen in *H. influenzae* is groot, zoals geïllustreerd wordt door het feit dat zes van de zeven mechanismen beschreven zijn (zie hoofdstuk 4). De splitsing van antimicrobiële resistentie in twee hoofdgroepen, genoemd "positieve functie-resistentie" en "persistentie" is ook van toepassing op de antibiotische resistentie in *H. influenzae*. Voorbeelden van positieve functie-resistentie zijn: door plasmiden overgedragen resistentie-mechanismen in *H. influenzae* tegen ampicilline, chloramphenicol, tetracycline en aminoglycosiden (beschreven in hoofdstuk 4). Om verspreiding van deze vorm van resistentie te voorkomen, zou men zich moeten richten op de ontwikkeling van nieuwe antibiotica, op vermindering van het toedienen van antibiotica in het algemeen en

op een effectief beleid ter voorkoming van verspreiding van deze plasmiden in het ziekenhuismilieu.

Voorbeelden van persistentie als een resistentie-mechanisme in *H. influenzae* zijn: chromosomale mutaties in penicilline-bindende eiwitten, verminderde permeabiliteit van *H. influenzae* voor chloramphenicol en thymine-auxotrophie (zie hoofdstuk 3). Klinische isolaten met deze vorm van resistentie worden vaak gekweekt gedurende behandeling met antibiotica bij patiënten met luchtweginfecties. Aangetoond is dat staken van de toediening van antibiotica via wegvallen van de selectiedruk bij deze isolaten dikwijls leidt tot een terugkeer naar het aanvankelijke pheno- en/of genotype. Persistentie kan b.v. voorkomen worden door het gebruik van meer dan één antibioticum, de toediening van antibiotica met een verschillend werkingsspectrum en een meer kritische keuze van antibiotica bij de individuele patiënt.

In **hoofdstuk 3** wordt een samenvatting gegeven van de werkzaamheid en het klinische gebruik van trimethoprim en/of co-trimoxazol bij de behandeling van infecties, veroorzaakt door zowel Gram-positieve als Gram-negatieve micro-organismen. Ook wordt een overzicht gegeven van de resistentie-mechanismen tegen trimethoprim. De voornaamste mechanismen zijn:

1. productie van een structureel veranderd dihydrofolaat reductase (DHFR), dat resistent is tegen trimethoprim.
2. overproductie van DHFR.

Opvallend is de diversiteit in DHFRs van Gram-positieve en Gram-negatieve micro-organismen. Bestudering van de moleculaire regulering en de biochemische eigenschappen van DHFRs heeft geleid tot nieuwe inzichten in de ontwikkeling en verspreiding van antibiotische resistentie-genen. De ontwikkeling van resistentie tegen trimethoprim in *Enterobacteriaceae*, *Salmonella* en *Shigella* is een groot probleem, speciaal in ontwikkelingslanden. Trimethoprim is ook een belangrijk antibioticum bij de behandeling van door *H. influenzae* veroorzaakte luchtweginfecties. Dit was de reden onderzoek te doen naar de genetische basis van en de biochemische resistentie-mechanismen tegen trimethoprim in *H. influenzae* (zie hoofdstukken 5, 6 en 7).

In **hoofdstuk 4** wordt een overzicht gegeven van de risicofactoren, de ziekten en de keuze van antibiotica bij infecties veroorzaakt door *H. influenzae*. De problemen bij de uitvoering van gevoeligheidsbepalingen van *H. influenzae* en de noodzaak om tot standaardisatie van MIC-bepalingen te komen, worden besproken. Verschillen in de samenstelling van de voedingsbodems en het bacteriële inoculum en het feit dat bepaalde antibiotica door bestanddelen in de voedingsbodems geïnhibeerd worden, zijn

van invloed op de betrouwbaarheid van MIC-bepalingen. Nauwkeurige opsporing van tegen ampicilline resistente, non-β-lactamase-producerende *H. influenzae* en van bacteriën, resistent tegen trimethoprim, wordt hierdoor bemoeilijkt.
De genetische en biochemische resistentie-mechanismen tegen vele antibiotica worden besproken. Hierbij komen aan de orde: resistentie tegen klinisch belangrijke antibiotica als ampicilline en verscheidene andere β-lactams, chloramphenicol en trimethoprim, alsmede resistentie tegen combinaties van antibiotica. Het voornaamste resistentie-mechanisme tegen ampicilline is de productie van een TEM-β-lactamase, dat zich bevindt op een 3 x 10^6 dalton sequentie van DNA [transposon A(TnA)]. TnA is waarschijnlijk afkomstig van plasmiden in andere Gram-negatieve micro-organismen. TEM-β-lactamase is ook de oorzaak van resistentie tegen ampicilline in meervoudig-resistente *H. influenzae*-stammen (zie hoofdstuk 8). Non-β-lactamase-producerende *H. influenzae*, die resistent zijn tegen ampicilline, worden steeds meer gerapporteerd. Het resistentie-mechanisme bij deze stammen wordt gevormd door een mutatie in de penicilline-bindende eiwitten, die een afnemende affiniteit voor verschillende β-lactam antibiotica tot gevolg heeft. De door een plasmide bewerkstelligde productie van chloramphenicol acetyltransferase (CAT) is de voornaamste oorzaak van resistentie tegen chloramphenicol (zie hoofdstuk 8). Moleculaire en biochemische mechanismen van trimethoprim-resistentie worden in de hoofdstukken 5, 6 en 7 behandeld.

De klinische gevolgen van het toenemende percentage antibiotische resistentie in *H. influenzae* worden besproken. De keuze van antibiotica bij de behandeling van infecties, veroorzaakt door *H. influenzae*, wordt toegelicht in de tabellen 4.1 en 4.2. Ampicilline blijft de eerste keus bij de behandeling van voor dit antibioticum gevoelige *H. influenzae*. Als alternatief voor de behandeling van meningitis kunnen 3e-generatie cephalosporines toegepast worden. Andere effectieve antibiotica voor de behandeling van longontsteking, middenoorontsteking, sinusitis en chronische luchtweginfecties zijn: amoxicilline-clavulanaat, cefuroxim en trimethoprim-sulfamethoxazol.

In **hoofdstuk 5** wordt verslag gedaan van het klonen van het gen, dat codeert voor resistentie tegen trimethoprim in *H. influenzae* en van een voorlopige karakterisering van het resistentie-mechanisme. Tien ongekapselde, tegen trimethoprim resistente (Tmp^r), *H. influenzae*-isolaten werden ontdekt. De MICs van deze stammen varieerden tussen de 10 μg/ml en > 200 μg/ml; gevoelige stammen hadden een MIC van < 0.5 μg/ml. Trimethoprim-resistentie kon zowel in conjugatie- als transformatie-experimenten overgedragen worden. Ofschoon de overdracht van resistentie in conjugatie-experimenten de aanwezigheid van plasmiden suggereerde, konden deze in de oorspronkelijke Tmp^r-stammen en in Tmp^r-transconjuganten niet aangetoond worden. Hieruit werd de conclusie getrokken, dat resistentie tegen trimethoprim in *H. influenzae* chromosomaal gecodeerd is en dat overdracht van resistentie-genen in conjugatie-experimenten tot stand komt via een

tot nog toe onbekend mechanisme. Over soortgelijke experimenten, die deze theorie ondersteunen, is al eerder gepubliceerd door Albritton et al. [1]. Het trimethoprim-resistentie-gen van stam R1047 (MIC 40 μg/ml) werd gekloond in een cosmide en recombinante plasmiden werden getransduceerd in *E. coli* HB101. Zes recombinante cosmide-klonen, die zowel resistent waren tegen ampicilline als tegen trimethoprim, werden op L-agar voedingsbodems geselecteerd. Transformatie-experimenten met plasmide-DNA leverden Tmp^r-transformanten op in een frequentie van 1×10^{-3}. Een 12.9-kb *Eco*RI fragment van één van de plasmiden werd gekloond in pGEM3. Het resulterende plasmide - pRGS7 genoemd - veroorzaakte nog steeds resistentie tegen trimethoprim. Een restrictie-enzym-map van pRGS7 werd ontwikkeld (figuur 5.1). Door middel van transformatie-experimenten met door restrictie-enzymen geknipt DNA van Tmp^r-stammen en met verschillende restrictie-enzym-fragmenten uit pRGS7, werd de positie bepaald van het gen, dat codeert voor resistentie tegen Tmp.

Een intragene *Xba*I-*Pst*I-probe werd gebruikt bij kolonie-hybridisatie en bij Southern-blot experimenten. De probe hybridiseerde zowel met gekapselde en ongekapselde Tmp^r *H. influenzae*-stammen alsook met gekapselde en ongekapselde trimethoprim-gevoelige (Tmp^s) *H. influenzae*-stammen. Er werd geen hybridisatie waargenomen met DNA van Tmp^r *Escherichia coli* dat dihydrofolaat reductase I, II en III genen bevatte, of met Tmp^r *Neisseria meningitidis*, *Neisseria gonorrhoeae* en *Pseudomonas cepacea*. Hieruit kan geconcludeerd worden dat de genen die voor resistentie tegen trimethoprim in deze micro-organismen coderen, in grote mate verschillen van het resistentie-gen tegen trimethoprim in *H. influenzae*. Dit is begrijpelijk, gezien de aanmerkelijke verschillen tussen bacteriële DHFRs in de aminozuur-sequentie en de nucleotide-sequentie (zie hoofdstuk 3). Southern-blot analyse met door middel van verschillende restrictie-enzymen geknipt DNA van isogene, Tmp^s- en Tmp^r-stammen toonde aan, dat de probe hybridiseerde met DNA-fragmenten van verschillende grootte bij gebruikmaking van *Dde*I en *Hin*fI. Uit deze gegevens werd geconcludeerd dat het verwerven van resistentie veroorzaakt wordt door een herschikking of een verandering in de nucleotiden-sequentie. De *Xba*I-*Pst*I-probe werd gebruikt in Southern-blot experimenten, om een antwoord te vinden op de vraag of de resistentie tegen trimethoprim chromosomaal gecodeerd is. Plasmide en chromosomaal DNA van meervoudig-resistente *H. influenzae*-isolaten uit Spanje, en van Tmp^r-transconjuganten van deze stammen (beide beschreven in hoofdstuk 8), werden gehybridiseerd met de *Xba*I-*Pst*I-probe. De probe hybridiseerde met het chromosomale DNA van alle Tmp^s- en Tmp^r-stammen en bleek geen overeenkomst te vertonen met plasmide DNA van Tmp^r-transconjuganten. Hierdoor werd de theorie, dat resistentie tegen trimethoprim chromosomaal gecodeerd is, ondersteund.

Vervolgens werd onderzoek gedaan naar het trimethoprim-resistentie-mechanisme in *H. influenzae*. Analyse van buitenmembraan eiwitprofielen en experimenten om vast te stellen in hoeverre trimethoprim in de bacterie penetreert, toonden geen verschillen aan

tussen Tmp^s en Tmp^r *H. influenzae*.
Metingen van DHFR-activiteit in het supernatant van het cel-sonicaat van Tmp^r-bacteriën, toonden aanzienlijk grotere activiteit dan metingen van DHFR-activiteit in Tmp^s-stammen. Uit deze gegevens kon geconcludeerd worden dat resistentie tegen trimethoprim in *H. influenzae* veroorzaakt wordt door overproductie van DHFR.

In **hoofdstuk 6** wordt de gedeeltelijke zuivering en karakterisering van DHFR uit een voor trimethoprim gevoelige stam (Tmp^s; stam MAP) en twee tegen trimethoprim resistente stammen (Tmp^r;stammen MAP/47 en MAP/42) van *H. influenzae* beschreven. De enzymen werden 650-3000-voudig gezuiverd door middel van een combinatie van ammonium-sulphaat fractionering, verdeling naar molecuul-grootte op een Sephadex G-75 kolom en affiniteit-chromatografie op een Matrix Gel Green A kolom. De totale enzym-activiteit van de electrophoretisch gezuiverde preparaten van MAP/47 en MAP/42 was zes- tot tienvoudig groter dan die verkregen uit MAP, terwijl de specifieke activiteit in eenheden/mg eiwit ongeveer gelijk was aan het eind van het zuiveringsproces. Dit bevestigde onze eerdere conclusie (hoofdstuk 5), dat resistentie tegen trimethoprim in *H. influenzae* veroorzaakt wordt door overproductie van chromosomaal gecodeerd DHFR.
De bepaling van de molecuul-grootte van de drie DHFRs door middel van SDS/PAGE toonde een eiwit met een molecuul-massa van 18.4 kDa. Michaelis-Menten-analyses met gezuiverde DHFR-fracties lieten zien dat de Km voor dihydrofolaat (7, 9 en 5 μM) en NADPH (2, 5 en 6 μM) voor alle drie enzymen gelijk was. Aanzienlijke verschillen werden echter ontdekt tussen de IC_{50} (de concentratie die nodig is voor een 50% remming van DHFR-activiteit) door trimethoprim van de Tmp^s-stam MAP (0.001 μM) en die van MAP/47 en MAP/42 (respectievelijk 0.1 μM en 0.3 μM). De IC_{50} door methotrexaat van MAP/42 DHFR was ook hoger (0.06 μM), vergeleken met de enzymen van MAP (0.008 μM) en MAP/47 (0.007 μM). Deze resultaten leken te bevestigen dat Tmp-resistentie in *H. influenzae* niet uitsluitend door overproductie van DHFR veroorzaakt wordt, maar ook door structurele veranderingen in de DHFRs van Tmp^r-stammen. Iso-electrische focusing-experimenten met de drie DHFR-fracties ondersteunden deze gegevens. DHFR van MAP/42 had namelijk een iso-electrisch punt (pI 7.6) dat verschillend was van de enzymen van MAP en MAP/47 (pI 7.3). "Peptide-mapping", gebruik makend van trypsine, toonde één peptide-fragment (7.9 kDa) in DHFR van MAP en MAP/47 en drie fragmenten (7.9, 9.6 en 12.5 kDa) in DHFR van MAP/42. Hieruit kan worden geconcludeerd dat er structurele en functionele verschillen bestaan tussen DHFRs in elke van de drie Tmp^s- en Tmp^r-stammen. Wij veronderstellen dat de genetische basis van resistentie tegen trimethoprim in *H. influenzae* overeenkomt met die, zoals beschreven bij mutanten van DHFR in *Escherichia coli* K12 [5]. Smith & Calvo hebben de moleculaire basis van resistentie tegen trimethoprim in mutanten van *Escherichia coli* bestudeerd [6]. Zij ontdekten dat de overproductie van DHFR in deze bacteriën veroorzaakt werd door een

mutatie in de promotor. Een mutatie in het structurele "fol"-gen was verantwoordelijk voor de verminderde affiniteit voor trimethoprim.

Hoofdstuk 7 behandelt het subklonen en de nucleotide-sequentie van een 1144 "base-pair" (bp) DNA-fragment van de *H. influenzae*-stam R1047, die resistent is tegen trimethoprim. Deze DNA-sequentie bevat twee genen die, respectievelijk, 480 en 561 bp bevatten. Het eerste gen codeert voor een eiwit met een molecuul-grootte van 17760 Da. De aminozuursequentie is voor 51.3% homoloog met DHFR van *E. coli* K12. De overeenkomst met andere prokaryotische DHFRs - zowel gevoelig voor als resistent tegen trimethoprim - varieerde tussen 34.9 en 49.1%. De molecuul-grootte van R1047 DHFR komt overeen met de berekende 18400 Da van gezuiverd DHFR (zie hoofdstuk 6). We vroegen ons af of de overproductie van veranderd DHFR in stam R1047 veroorzaakt kon zijn door mutaties in het structurele "fol"-gen en in het promotorgebied. De grote verschillen tussen DHFR van *H. influenzae* R1047 en andere DHFRs maken het echter moeilijk om vast te stellen of veranderingen in het structurele gen functionele veranderingen in DHFR weergeven of dat ze een gevolg zijn van normale variabiliteit tussen DHFRs van verschillende micro-organismen.
De vergelijking van het structurele gen en het daarbij behorende promotorgebied van R1047 DHFR met DHFR-sequenties van andere micro-organismen - zowel Gram-positieve als Gram-negatieve - toont enkele interessante aspecten. De aminozuren die betrokken zijn bij de interactie van DHFR met NADPH òf met foliumzuur antagonisten, zijn zeer goed geconserveerd in *H. influenzae* R1047 DHFR zoals gebruikelijk is bij andere prokaryotische DHFR-sequenties (figuur 7.3, hoofdstuk 7). De vervanging van een enkel aminozuur in één van de bovengenoemde gebieden, kan tot belangrijke wijzigingen leiden in de binding van foliumzuur antagonisten en in de kinetische eigenschappen van DHFR. Een dergelijke verandering heeft mogelijk plaats gevonden op positie 121 van het DHFR-gen. Bij vergelijking van het promotorgebied van het R1047 "fol"-gen en verscheidene andere DHFR-promotor-sequenties blijkt de -10 sequentie volledig identiek te zijn aan de -10 sequentie in *E. coli* K12, terwijl de -35 sequentie in 5 van de 6 nucleotiden identiek is aan het -35 gebied in *E. coli* 1810, die een sterke overproductie van DHFR te zien geeft. De aanwezigheid van een T in positie -34 van de -35 sequentie kan belangrijk zijn, aangezien beschreven is, dat een conversie van C naar T op deze plaats kan resulteren in een twaalf- tot vijftienvoudig toegenomen synthese van "fol" mRNA in gemuteerde *E. coli*-stammen. Vergelijkbare veranderingen in het -35 en/of -10 gebied kunnen verantwoordelijk zijn voor de overproductie van DHFR in *H. influenzae* R1047.

Het is onze bedoeling in de toekomst de gen-regulering van R1047 "fol" nader te bestuderen door middel van mutagenese-experimenten. Daartoe willen wij de A op positie -35 door een T vervangen, hetgeen resulteert in een TTG-sequentie. Aangetoond is, dat

deze sequentie karakteristiek is voor krachtig werkende Gram-negatieve promotors. Derhalve valt te verwachten dat deze omzetting zal resulteren in een aanzienlijke toeneming van DHFR. Tevens willen we de sequentie analyseren van het DHFR-gen en van het promotorgebied van de Tmp^r-stam R1042 en de Tmp^s-stam R906. Hierbij zal gebruik gemaakt worden van de polymerase kettingreactie (polymerase chain reaction, PCR).

Het tweede gen op de complementaire streng bevat 561 nucleotiden. Een analyse van het structurele gen toont 62,9% overeenkomst met de eerste 187 aminozuren van de N-terminale sequentie van het proBA-gen. Het gebied 5' van het proB-gen bevat een "ribosome binding site", en -10 en -35-promotor-sequenties (figuur 8.2B). Het proB-gen codeert voor γ-glutamyl-kinase. Dit enzym speelt een belangrijke rol bij de bacteriële proline-synthese. Proline is betrokken bij de bacteriële eiwit-synthese en dient als bron van koolstof en stikstof. Het is interessant dat de proBA-positie op de genmap van *E. coli* (op 6 min) ver verwijderd is van het DHFR "fol A"-gen (op 1 min), terwijl deze twee genen naast elkaar liggen in het *H. influenzae*-genoom. Onduidelijk is vooralsnog of dit enige functionele betekenis heeft.

Het onderwerp van **hoofdstuk 8** is de genetische verwantschap van de determinanten, verantwoordelijk voor resistentie tegen antibiotica in meervoudig resistente *H. influenzae*. Resistentie van *H. influenzae*-bacteriën tegen ampicilline, chloramphenicol en tetracycline, al dan niet gepaard gaand met resistentie tegen trimethoprim, kanamycine, sulfamethoxazole en streptomycine, is uitgegroeid tot een groot probleem in Spanje en enkele andere Europese landen [2, 3]. Tien meervoudig-resistente *H. influenzae*-isolaten werden onderzocht, teneinde een antwoord te krijgen op twee vragen: 1) wordt meervoudige resistentie in *H. influenzae* uit verschillende geografische gebieden veroorzaakt door één enkele plasmide of meerdere plasmiden en 2) zijn de antibiotische resistentie-genen uit verschillende geografische gebieden gelijk of verschillend. Antibiotische resistentie tegen ampicilline, chloramphenicol, tetracycline, kanamycine en sulfamethoxazole wordt gecodeerd door conjugatieve plasmiden met moleculaire massa's van 38-50 MDa. In tegenstelling hiermee was de resistentie tegen trimethoprim niet gecodeerd door plasmiden, maar chromosomaal bepaald (zie ook hoofdstuk 5). Door alle transconjugante stammen werd chloramphenicol acetyltransferase (CAT) geproduceerd en op één na alle stammen produceerden TEM-1 β-lactamase. Transconjuganten die geselecteerd waren op chloramphenicol en erythromycine vertoonden alle 8-50 maal meer kanamycine-phosphotransferase-activiteit dan de voor kanamycine gevoelige stam MAP. Restrictie-enzym digestie van de geïsoleerde plasmiden bracht punten van overeenkomst aan het licht, maar er bleek ook een zekere mate van heterogeniteit, afhankelijk van het gebruikte restrictie-enzym. Southern-blot analyse van deze plasmiden, met gebruik van diverse antibiotische resistentie-probes, liet verschillende combinaties van hybridisatie-patronen zien, afhankelijk van het gebruikte restrictie-enzym. De theorie dat resistentie tegen

meerdere antibiotica in *H. influenzae* niet ontstaat door de verwerving van een enkele R-plasmide, werd door deze experimenten ondersteund. Uit dit onderzoek kan worden geconcludeerd dat plasmide-resistentie (tegen ampicilline, chloramphenicol en kanamycine) en chromosomale resistentie (tegen trimethoprim) van verschillende herkomst zijn.

REFERENTIES

1 **Albritton W.L., Setlow J.K., Slaney L.** *Transfer of Haemophilus influenzae chromosomal genes by cell-to-cell contact.* J Bacteriol 1982;152:1066-1070.

2 **Campos J., García-Tornel S., Gairi Tahull J.M.** *Invasive infections caused by multiply resistant Haemophilus influenzae type b.* J Pediatr 1984;104:162-163.

3 **Campos J., García S., Gairi Tahull J.M., Fábregues I.** *Multiply resistant Haemophilus influenzae type b causing meningitis:comparative clinical and laboratory study.* J Pediatr 1986;108:897-902.

4 **O'Brien T.F. and members of Task Force 2** *Resistance of Bacteria to antimicrobial agents: report of task force 2.* Rev Infect Dis 1987;9:S244-S260.

5 **Sheldon R.** *Altered dihydrofolate reductase in fol regulatory mutants of Escherichia coli K12.* Molec Gen Genet 1977;151:215-219.

6 **Smith D.R., Calvo J.M.** *Nucleotide sequence of dihydrofolate reductase genes from trimethoprim-resistant mutants of Escherichia coli.* Mol Gen Genet 1982;187:72-78.

7 **Workshop Rijksinstituut voor Volksgezondheid en Milieuhygiëne.** *Veterinair antibioticagebruik en volksgezondheid.* Bilthoven 22 maart 1990.

ACKNOWLEDGMENTS/DANKWOORD

This thesis would not have been completed without the help of many people. The main body of the work was performed in the Department of Pediatric Infectious Diseases, University of Washington, Seattle, USA under the stimulating guidance of Professor Dr. A.L. Smith.
The second part of the study was carried out in the Departments of Pediatrics and Clinical Microbiology, Sophia Children's Hospital and Erasmus University, Rotterdam, The Netherlands. The study was financially supported by grants from the Sophia Foundation for Scientific Research and the American Cystic Fibrosis Foundation.

Professor Dr. A.L. Smith, Dear Arnie, I am deeply grateful for your friendship and continuing support. I will not forget your good example as an eminent scientist and clinician.

I would like to acknowledge the friendship and technical assistance of Sandy Burchett, Jane Burns, José Campos, Donald Chaffin, Keith English, Laura Heggan, Laura Johnson, Meta Kuehn, Dave Lewis, Paul Mendelman, Steve Moseley, Craig Rubens, Allan Weber, Chris Wilson and Keith Wong. They all and many others contributed to the uniquely stimulating atmosphere within the laboratory in which I was privileged to work.

Het onderzoek werd voortgezet in Rotterdam.

Professor Dr. H.J. Neijens creëerde op enthousiaste wijze de mogelijkheden voor verder onderzoek, beste Herman, de wijze waarop wij de afgelopen jaren in goede vriendschap hebben samengewerkt in onderzoek en kliniek binnen het jonge subspecialisme Pediatrische Infectieziekten belooft veel goeds voor de toekomst.

Professor Dr. H.K.A. Visser, beste Henk, de wijze waarop jij als leermeester mijn vorming als clinicus en onderzoeker hebt gestimuleerd, waardeer ik zeer.

Professor Dr. M.F. Michel, beste Mark, ik ben je zeer erkentelijk voor de goede samenwerking en de gastvrijheid die jouw afdeling mij biedt.

Professor Dr. W.P.M. Hoekstra dank ik voor zijn bereidwilligheid dit proefschrift van kritische kanttekeningen te voorzien.

Dr. W.H.F. Goessens, beste Wil, je vriendschap en steun bij het onderzoek stel ik zeer op prijs.

M. Sluijter, beste Marcel, de zelfstandige, enthousiaste en consciëntieuze wijze waarop jij experimenten uitvoert, waardeer ik buitengewoon.

Jacqueline Dito, ik wil je bedanken voor je meer dan voortreffelijke secretariële ondersteuning bij de totstandkoming van dit proefschrift.

Willem van Smaalen wil ik bedanken voor zijn deskundige en creatieve hulp bij de vormgeving van dit proefschrift.

Hannie Tazelaar-de Groot ben ik dankbaar voor haar correctie- en vertaalwerk (mijn tante staat op haar strepen).

Auke Beishuizen wil ik bedanken voor alle tijd, die hij besteed heeft aan de lay-out van de figuren.

Joop van Dijk wil ik bedanken voor het omslagontwerp.

Riet Visser-Vermeer en Jacqueline de Vogel-Augustijn bedank ik voor hun efficiënte ondersteuning van dit onderzoek.

Dr. G. Dzoljic-Danilovic, beste Gordena, ik ben je zeer dankbaar voor je vriendschap en voor je grote inzet bij onze samenwerking.

Mijn collega's in de pediatrisch intensive care groep, Hein Brackel, Arda Derksen, Liesbeth van Essen, Jan Hazelzet, Carlien Kransberg, Anja Verberne, Edwin van der Voort en Maarten Witsenburg wil ik bedanken voor het waarnemen van mijn diensten tijdens de afronding van dit proefschrift.

Mijn moeder ben ik dankbaar voor haar grote inzet en steun door de jaren heen.

Valerie, zonder je geduld en nooit aflatende steun zou dit proefschrift niet tot stand gekomen zijn. Ik hoop na de promotie meer tijd met jou, Steven, Mark, Arjan en Ewout te kunnen doorbrengen.

Capelle a/d IJssel,
juni 1991.

Ronald

CURRICULUM VITAE

Ronald de Groot

29 mei 1948:	Geboren te Rotterdam.
juni 1967:	Diploma Gymnasium α Gymnasium Erasmianum, Rotterdam.
1968:	Colloquim Doctum.
1968-1975:	Studie Geneeskunde Erasmus Universiteit, Rotterdam.
feb. 1971-juli 1971:	Keuze-practicum: Afdeling Psychiatrie. Onderwerp: Evaluatie onderzoek van daghospitaal-behandeling.
sept. 1973:	Doctoraal examen Geneeskunde. Erasmus Universiteit, Rotterdam.
feb. 1975:	Artsexamen. Erasmus Universiteit, Rotterdam.
feb. 1975-juni 1975:	Assistentschap Verloskunde en Gynaecologie. St. Clara Ziekenhuis, Rotterdam.
juni 1975-sept. 1976:	Assistentschap Chirurgie. St. Laurens Ziekenhuis, Breda.
okt. 1976-dec. 1976:	Nationale Cursus Tropische Geneeskunde en Hygiëne. Koninklijk Instituut voor de Tropen, Amsterdam.

feb. 1977-juni 1977: Tropenarts.
St. Louis Hospital, Zonkwa, Kaduna State, Nigeria.

juli 1979-april 1983: Opleiding Kindergeneeskunde.
Sophia Kinderziekenhuis, Rotterdam.

april 1983-juni 1985: Chef-de-Clinique Kindergeneeskunde.
Sophia Kinderziekenhuis, Rotterdam.

juli 1985-juli 1988: Research en Klinisch fellowship in Pediatrische Infectieziekten.
Children's Hospital and Medical Center, Seattle, USA.
Onderzoek:
Antibiotische resistentie in *Haemophilus influenzae*.
Pharmacokinetiek van antibiotica bij patienten met Cystic Fibrosis.

aug. 1988-heden: Universitair Docent aan de Erasmus Universiteit, Rotterdam, staflid in het Sophia Kinderziekenhuis, afdeling Kindergeneeskunde, subafdeling Infectie- en Immuunziekten.

juli 1985-heden: Promotie-onderzoek:
Afdeling Pediatric Infectious Diseases, Children's Hospital and Medical Center, Seattle, USA. (o.l.v. Prof. Dr. A.L. Smith) en Afdeling Kindergeneeskunde, Sophia Kinderziekenhuis, Rotterdam (o.l.v. Prof. Dr. H.J. Neijens).
Onderwerp: Antibiotic resistance in *Haemophilus influenzae*.

LIST OF PUBLICATIONS

(Original Articles, Book Chapters, and Reviews)

1 **De Groot R., Mettau J.W., Dzoljic-Danilovic G., Michel M.F.** *Sepsis en meningitis bij pasgeborenen en jonge zuigelingen veroorzaakt door Streptokokken van groep B (Streptococcus agalactiae).* Ned Tijdschr Geneeskd 1983;127:2175-2179.

2 **Lafeber H.N., van der Voort E., de Groot R.** *Haemorrhagic shock and encephalopathy syndrome.* Lancet 1983;II:795.

3 **De Groot R., Essed C.E., Gaillard J.L.J., Mettau J.W., de Villeneuve V.H.** *Primary restrictive foramen ovale.* Eur J Pediatr 1984;141:248-249.

4 **De Groot R., Oranje A.P., Vuzevski V.D., Mettau J.W.** *Toxic epidermal necrolysis probably due to Klebsiella pneumoniae* sepsis. Dermatologica 1984;169:88-90.

5 **De Groot R., Oranje A.P., Vuzevski V.D.** *"Staphylococcal scalded skin"-syndroom.* Ned Tijdschr Geneeskd 1984;128:1459-1463.

6 **De Groot R., Oranje A.P., van der Heyden A.J., Vuzevski V.D., Schaap G.J.P., van Joost Th.** *Rickettsia conorii infection complicated by supraventricular tachycardia in a ten year-old child.* Acta Leidensia 1984;52:45-52.

7 **Oranje A.P., De Groot R.** *Toxic epidermal necrolysis probably due to Klebsiella pneumoniae sepsis.* Dermatologica 1984;169:88-90.

8 **Oranje A.P., De Groot R.** *Toxic epidermal necrolysis.* Pediatr Dermatol 1985;3:83.

9 **De Zegher F.E., Diepersloot R.J.A., Neijens H.J., de Groot R.** *Infecties met respiratoir syncitieel virus bij zuigelingen en kleuters.* Ned Tijdschr Geneeskd 1985;129:2157-2161.

10 **Van Steensel-Moll H.A., de Groot R., Neijens H.J., den Hollander J.C., Meradji M.** *Bronchial carcinoid tumor in a 12-year-old child.* Pediatr Pulmonol 1986;2:110-113.

11 **Oranje A.P., de Groot R.** *Mucocutane syndromen.* In: Oranje A.P.,ed. Enkele aspecten van de kinderdermatologie. Nieuwegein: Glaxo 1987:131-144.

12 **De Groot R., Smith A.L.** *Antibiotic pharmacokinetics in cystic fibrosis: differences and clinical significance.* Clin Pharmacokinet 1987;13:228-253.

13 **Mendelman P.M., Chaffin D.O., Musser J.M., de Groot R., Serfass D.A., Selander R.K.** *Genetic and phenotypic diversity among ampicillin-resistant, non-β-lactamase-producing, nontypeable Haemophilus influenzae isolates.* Infect Immun 1987;55:2585-2589.

14 **De Groot R., Campos J., Moseley S.L., Smith A.L.** *Molecular cloning and mechanism of trimethoprim resistance in Haemophilus influenzae.* Antimicrob Agents Chemother 1988;32:477-484.

15 **Oranje A.P., Vuzevski V.D., de Groot R., Prins M.E.F.** *Congenital self-healing non-Langerhans cell histiocytosis.* Eur J Pediatr 1988;148:29-31.

16 **De Groot R., Glover D., Clausen C., Smith A.L., Wilson C.B.** *Bone and joint infections caused by Kingella kingae : six cases and review of the literature.* Rev Infect Dis 1988;10:998-1004.

17 **Campos J., Chanyangam M., De Groot R., Smith A.L., Tenover F.C., Reig R.** *Genetic relatedness of antibiotic resistance determinants in multiply resistant Haemophilus influenzae.* J Infect Dis 1989;160:810-817.

18 **Neijens H.J., De Groot R., Dzoljic-Danilovic G.** *Longinfecties met atypische aspecten.* Tijdschr Kindergeneeskd 1990;58:23-29.

19 **De Groot R., Hack B.D., Weber A., Chaffin D., Ramsey B., Smith A.L.** *Pharmacokinetics of ticarcillin in patients with cystic fibrosis: a controlled prospective study.* Clin Pharmacol Ther 1990;47:73-78.

20 **Smit M.J.M., de Groot R., van Dongen J.J.M., van der Voort E., Neijens H.J., Whitfield L.R.** *Trimetrexate efficacy and pharmacokinetics during treatment of refractory Pneumocystis carinii pneumonia in an infant with severe combined immunodeficiency disease.* Pediatr Infect Dis 1990;9:212-214.

21 **Oranje A.P., de Groot R.** *Exanthemen.* In: Boer J., Hulsmans R.F.H.J., van de Kerkhof P.C.M., van der Meer J.B., Oranje A.P., Wuite J.,eds. 10e Nascholingscursus van de Nederlandse Vereniging voor Dermatologie en Venereologie. Mijdrecht: Zyma B.V. 1990:17-23.

22 **De Groot R., Rümke H.C., Roord J.J.** *Bacteriële meningitis bij jonge kinderen.* Ned Tijdschr Geneeskd 1990;58:193-200.

23 **de Bel C.E., de Groot R., Schrander J.J.P., Rothbarth Ph.H.** *Congenitale cytomegalovirus-infectie.* Tijdschr Kindergeneeskd 1990;58:211-217.

24 **v.d. Anker J.N., de Groot R., v.d. Heijden B.J.** *Use of antibiotics in neonates weighing less than 1200 G.* Pediatr Infect Dis J 1990;9:752-753.

25 **Neijens H.J., Sinaasappel M., de Groot R., de Jongste J.C., Overbeek S.E.** *Cystic fibrosis, pathophysiological and clinical aspects.* Eur J Pediatr 1990;149:742-751.

26 **Van der Willigen A.H., de Groot R., Oranje A.P.** *Infecties.* In: Oranje A.P.,ed. Aspecten van de kinderdermatologie. Lochem: de Tijdstroom 1990:41-68.

27 **Oranje A.P., de Groot R.** *Exanthemen.* In: Oranje A.P.,ed. Aspecten van de kinderdermatologie. Lochem: de Tijdstroom 1990:97-106.

28 **de Groot R., Oranje A.P.** *Mucocutane syndromen.* In: Oranje A.P.,ed. Aspecten van de kinderdermatologie. Lochem: de Tijdstroom 1990;107-121.

29 **De Groot R., Chaffin D.O., Kuehn M., Smith A.L.** *Trimethoprim resistance in Haemophilus influenzae is due to altered dihydrofolate reductase(s).* Biochem J 1991;274:657-662.

30 **Smit M.J.M., de Groot R., van Dongen J.J.M., Hazelzet J.A., Sluiters J.F., Neijens H.J.** *Pneumocystis carinii-pneumonie bij patiënten met een ernstige gecombineerde immunodeficiëntie.* Ned Tijdschr Geneeskd 1991;135:24-26.

31 **Goeteyn M., Oranje A.P., Vuzevski V.D., de Groot R., van Suijlekom-Smit L.W.A.** *Ichthyosis, exocrine pancreatic insufficiency, impaired neutrophil chemotaxis, growth redardation and metaphyseal dysplasia ("Shwachman syndrome").* Arch Dermatol 1991;127:225-230.

32 **Neijens H.J., de Groot R., Dzoljic-Danilovic G.** *Haemophilus influenzae type b-infecties bij kinderen.* Ned Tijdschr Geneeskd 1991;135:13-16.

33 **Van Dongen J.J.M., de Groot R., Comans-Bitter W.M., Neijens H.J.** *T cell receptor γ-δ expression in healthy children and children with ataxia teleangiectasia.* In: Chapel H.M., Levinsky R.J., Webster A.D.B.,eds. Progress in immune deficiency III. London: Royal Society of Medicine Services 1991:305-306.

34 **Weber A., de Groot R., Ramsey B., Williams-Warren J., Smith A.L.** *Probenecid pharmacokinetics in cystic fibrosis.* Developm Pharmacol 1991, in press.

35 **De Groot R., Dzoljic-Danilovic G., van Klingeren B., Goessens W.H.F., Neijens H.J.** *Antibiotic resistance in Haemophilus influenzae: mechanisms, clinical importance and consequences for therapy.* Eur J Pediatr 1991, in press.

36 **Koorevaar C.Th., Scherpenzeel P.G.N., de Groot R., Neijens H.J., Derksen-Lubsen G., Dzoljic-Danilovic G.** *Childhood meningitis caused by Enterococci and Streptococci viridans.* Accepted for publication.

37 **De Groot R., Sluijter M., Smith A.L., Goessens W.H.F.** *Nucleotide sequence of a trimethoprim-resistant chromosomally encoded dihydrofolate reductase from Haemophilus influenzae.* Submitted for publication.

38 **De Groot R., van Dongen J.J.M., Groothuis D.G., Hooijkaas H., van Steensel-Moll H.A., Neijens H.J.** *Disseminated Mycobacterium gordonae infection in a child without an evident immunodeficiency.* Submitted for publication.

39 **Boot J.M., Oranje A.P., de Groot R., Tan Y., Stolz E.** *Congenital syphilis.* Submitted for publication.

It doesn't take big bucks to do science,
not everything has been discovered,
and it's important to have fun.

(Donald Huffman)

Stellingen behorende bij het proefschrift

ANTIBIOTIC RESISTANCE IN HAEMOPHILUS INFLUENZAE

Rotterdam, 19 juni 1991

Ronald de Groot

1. Overdracht van resistentie tegen trimethoprim in Haemophilus influenzae via conjugatie-experimenten wordt niet gemedieerd door plasmiden.

2. Resistentie tegen trimethoprim in Haemophilus influenzae wordt veroorzaakt door overproductie van structureel en functioneel veranderde dihydrofolaat reductase(s).

3. Overproductie van dihydrofolaat reductase in voor trimethoprim resistente Haemophilus influenzae wordt waarschijnlijk veroorzaakt door mutaties in het -35 promotorgebied.

4. Het veterinaire gebruik van antibiotica dient sterk te worden beperkt.

5. De snelle klaring van geneesmiddelen bij patiënten met cystic fibrosis wordt veroorzaakt door gegeneraliseerde inductie van enzymen in lever en nier.

6. Dosering van geneesmiddelen met een lage serumeiwit binding dient bij preterme pasgeborenen gebaseerd te zijn op meting van de glomerulaire filtratiesnelheid.

7. Het gebruik van de Swan-Ganz catheter in de pediatrische intensive care is niet in het belang van de individuele patiënt.

8. In ongeveer 0,25% van alle ziekenhuisopnamen leidt medisch laakbaar handelen tot de dood.

9. Medisch handelen dient te worden gevalideerd; dit geldt ook voor bedrust.

10. De kwaliteit van klinisch wetenschappelijk onderzoek in Nederland zal verbeteren als men meer plaats inruimt voor researchverpleegkundigen.

11. Routine laboratoriumonderzoek is niet geïndiceerd.

12. Op het gebied van de foetale fysiologie kan de mens nog veel van de zeehond leren.

13. Het is zowel culinair als chirurgisch verwerpelijk de tafel voortijdig te verlaten.

14. Koolstofatomen kunnen zich niet alleen vormen tot diamanten en grafiet, doch ook tot buckyballs.