Bacteriophages

■ Bacteriophages, or simply phages, are viruses that infect bacteria. They possess a protein shell surrounding the phage genome, which with few exceptions is composed of DNA. A bacteriophage attaches to specific receptors on its host bacteria and injects its genome through the cell wall. This forces the host cells to synthesize more bacteriophages. The host cell lyses at the end of this reproductive phase. So-called temperate bacteriophages lysogenize the host cells, whereby their genomes are integrated into the host cell chromosomes as the so-called prophage. The phage genes are inactive in this stage, although the prophage is duplicated synchronously with host cell proliferation. The transition from prophage status to the lytic cycle is termed spontaneous or artificial induction. Some genomes of temperate phages may carry genes which have the capacity to change the phenotype of the host cell. Integration of such a prophage into the chromosome is known as lysogenic conversion. ■

Definition

Bacteriophages are viruses the host cells of which are bacteria. Bacteriophages are therefore obligate cell parasites. They possess only one type of nucleic acid, either DNA or RNA, have no enzymatic systems for energy supply and are unable to synthesize proteins on their own.

Morphology

Similarly to the viruses that infect animals, bacteriophages vary widely in appearance. Fig. 3.**25a** shows a schematic view of a T series coli phage. Research on these phages has been particularly thorough. Fig. 3.**25b** shows an intact T phage next to a phage that has injected its genome.

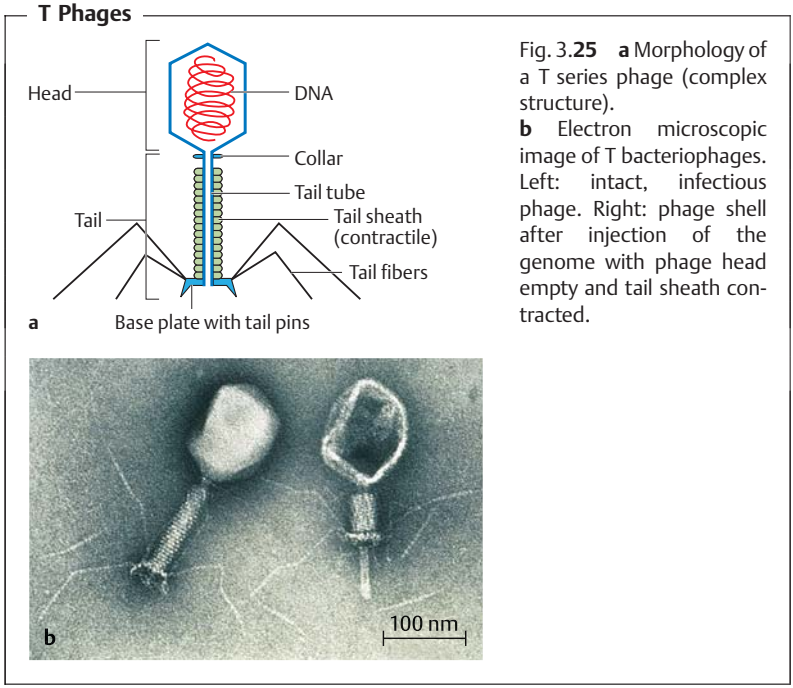

Fig. 3.**25** **a** Morphology of a T series phage (complex structure). **b** Electron microscopic image of T bacteriophages. Left: intact, infectious phage. Right: phage shell after injection of the genome with phage head empty and tail sheath contracted.

Composition

Phages are made up of protein and nucleic acid. The proteins form the head, tail, and other morphological elements, the function of which is to protect the phage genome. This element bears the genetic information, the structural genes for the structural proteins as well as for other proteins (enzymes) required to produce new phage particles. The nucleic acid in most phages is DNA, which occurs as a single DNA double strand in, for example, T series phages. These phages are quite complex and have up to 100 different genes. In spherical and filamentous phages, the genome consists of single-stranded DNA (example: ΦX174). RNA phages are less common.

Reproduction

The phage reproduction process involves several steps (Fig. 3.**26**).

- **Adsorption.** Attachment to cell surface involving specific interactions between a phage protein at the end of the tail and a bacterial receptor.

Lytic Phage Reproduction and Lysogenization

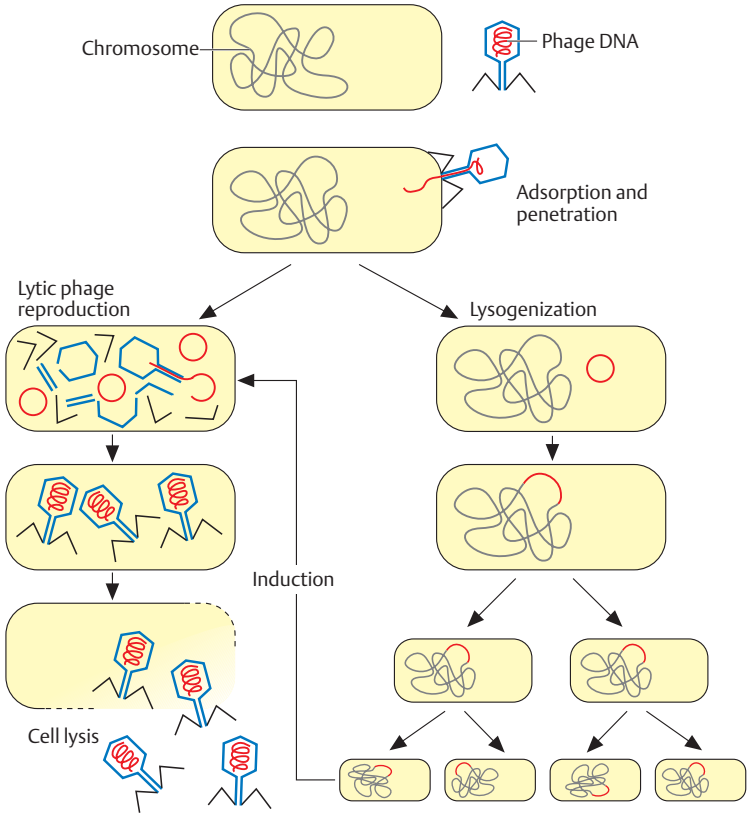

Fig. 3.**26** Injection of the phage genome is followed either by direct intracellular (lytic) phage reproduction or lysogenization of the host cell. In the lysogenization process, the phage DNA is integrated into the host cell chromosome and replicated together with it in the process of cell fission.

Release of Phages from the Host Cell

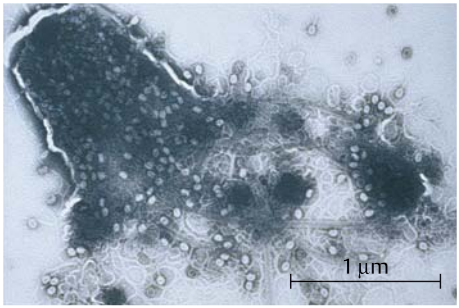

Fig. 3.**27** At the end of the phage maturation process, the host cell is lysed to release the new phages. Lysis occurs by a phage-encoded murein hydrolase, which gains access to the murein through membrane channels formed by the phage-encoded protein holin.

- **Penetration.** Injection of the phage genome. Enzymatic penetration of the wall by the tail tube tip and injection of the nucleic acid through the tail tube.

- **Reproduction**. Beginning with synthesis of early proteins (zero to two minutes after injection), e.g., the phage-specific replicase that initiates replication of the phage genome. Then follows transcription of the late genes that code for the structural proteins of the head and tail. The new phage particles are assembled in a maturation process toward the end of the reproduction cycle.

- **Release.** This step usually follows the lysis of the host cell with the help of murein hydrolase coded by a phage gene that destroys the cell wall (Fig. 3.**27**).

Depending on the phage species and milieu conditions, a phage reproduction cycle takes from 20 to 60 minutes. This is called the **latency period**, and can be considered as analogous to the generation time of bacteria. Depending on the phage species, an infected cell releases from 20 to several hundred new phages, which number defines the **burst size**. Thus phages reproduce more rapidly than bacteria. In view of this fact, one might wonder how any bacteria have survived in nature at all. It is important not to forget that cell population density is a major factor determining the probability of finding a host cell in the first place and that such densities are relatively small in nature. Another aspect is that only a small proportion of phages reproduce solely by means of these lytic or vegetative processes. Most are temperate phages that lysogenize the infected host cells.

Lysogeny

Fig. 3.**26** illustrates the **lysogeny** of a host cell. Following injection of the phage genome, it is integrated into the chromosome by means of region-specific recombination employing an integrase. The phage genome thus integrated is called a **prophage**. The prophage is capable of changing to the vegetative state, either spontaneously or in response to induction by physical or chemical noxae (UV light, mitomycin). The process begins with excision of the phage genome out of the DNA of the host cell, continues with replication of the phage DNA and synthesis of phage structure proteins, and finally ends with host cell lysis. Cells carrying a prophage are called **lysogenic** because they contain the genetic information for lysis. Lysogeny has advantages for both sides. It prevents immediate host cell lysis, but also ensures that the phage genome replicates concurrently with host cell reproduction.

Lysogenic conversion is when the phage genome lysogenizing a cell bears a gene (or several genes) that codes for bacterial rather than viral processes. Genes localized on phage genomes include the gene for diphtheria toxin, the gene for the pyrogenic toxins of group A streptococci and the *cholera toxin* gene.

The Importance of the Bacteriophages

Biological research	Bacteriophages are often used as models in studies of fundamental biological processes: DNA replication, gene expression, gene regulation, viral morphogenesis, studies of the details, and function of supramolecular structures
Genetic engineering	Vectors for gene cloning, adjuvants in sequencing
Therapy and prevention	An older concept now receiving increased attention. Administration of suitable phage mixtures in therapy and prevention of gastrointestinal infections. In animal husbandry, a number of phages that attack only EHEC (enterohemorrhagic *E. coli*) are used against EHEC infections
Epidemiology	Bacterial typing. Strains of a bacterial species are classified in phagovars (syn. lysotypes) based on their sensitivity to typing bacteriophages. Recognition of the bacterial strain responsible for an epidemic, making it possible to follow up the chain of infection and identify the infection sources. This typing method has been established for *Salmonella typhi*, *Salmonella paratyphi B*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and other bacteria, although it is now increasingly being replaced by new molecular methods, in particular DNA typing

The Principles of Antibiotic Therapy

Specific antibacterial therapy refers to treatment of infections with anti-infective agents directed against the infecting pathogen. The most important group of anti-infective agents are the antibiotics, which are products of fungi and bacteria (*Streptomycetes*). Anti-infective agents are categorized as having a broad, narrow, or medium spectrum of action. The efficacy, or effectiveness, of a substance refers to its bactericidal or bacteriostatic effect. Anti-infective agents have many different mechanisms of action. Under the influence of sulfonamides and trimethoprim, bacteria do not synthesize sufficient amounts of tetrahydrofolic acid. All betalactam antibiotics irreversibly block the biosynthesis of murein. Rifamycin inhibits the DNA-dependent RNA polymerase (transcription). Aminoglycosides, tetracyclines, and macrolides block translation. All 4-quinolones damage cellular DNA topology by inhibiting bacterial topoisomerases. Due to their genetic variability, bacteria may develop resistance to specific anti-infective agents. The most important resistance mechanisms are: inactivating enzymes, resistant target molecules, reduced influx, increased efflux. Resistant strains (problematic bacteria) occur frequently among hospital flora, mainly *Enterobacteriaceae*, pseudomonads, staphylococci, and enterococci. Laboratory resistance testing is required for specific antibiotic therapy. Dilutions series tests are quantitative resistance tests used to determine the minimum inhibitory concentration (MIC). The disk test is a semiquantitative test used to classify the test bacteria as resistant or susceptible. In combination therapies it must be remembered that the interactions of two or more antibiotics can give rise to an antagonistic effect. Surgical chemoprophylaxis must be administered as a short-term antimicrobial treatment only.

Definitions

Specific **antibacterial therapy** designates treatment of infections with **anti-infective agents** directed against the infecting pathogen (syn. **antibacterial chemotherapeutics, antibiotics**). One feature of these pharmaceuticals is "selective toxicity," that is, they act upon bacteria at very low concentration levels without causing damage to the macroorganism. The most important group of anti-infective agents is the **antibiotics**. These natural substances are produced by fungi or bacteria (usually *Streptomycetes*). *The term "antibiotic" is often used in medical contexts to refer to all antibacterial pharmaceuticals, not just to antibiotics in this narrower sense.* Fig. 3.**28** illustrates

Interactions: Anti-Infective Agent/Bacterium/Host Organism

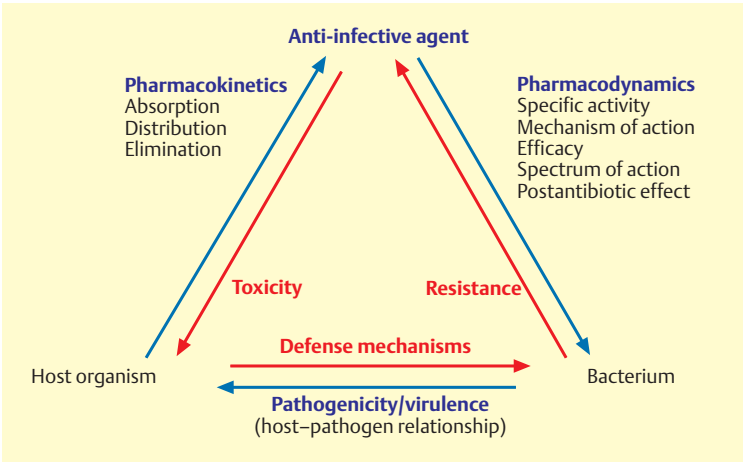

Fig. 3.**28** Interactions between the anti-infective agent and host organism are characterized by the terms pharmacokinetics and toxicity; interactions between the anti-infective agent and the bacterial pathogen are characterized in terms of pharmacodynamics and resistance.

the relations between an anti-infective agent, the host organism, and a bacterial pathogen. Table 3.**4** lists frequently used anti-infective agents. The most important groups (cephalosporins, penicillins, 4-quinolones, macrolides, tetracyclines) are in bold print. Fig. 3.**29** presents the basic chemical structures of the most important anti-infective agents.

Table 3.**4** Frequently Used Anti-Infective Agents

Class/active substance	Remarks
Aminoglycoside/aminocyclitol antibiotics	
(dihydro)streptomycin	For treatment of tuberculosis
neomycin, paramomycin	Only for oral or topical application
kanamycin	Parenteral administration; resistance frequent
gentamicin, tobramycin, amikacin, netilmicin, sisomicin	Newer aminoglycosides; broad spectrum; no effect on streptococci and enterococci; ototoxicity and nephrotoxicity; control of serum levels during therapy
spectinomycin	Against penicillinase-positive gonococci, in urogenital gonorrhea
Carbacephems	Betalactams structured like cephalosporins, but with a C atom instead of sulfur in the second ring system (see fig. 3.**29**, p. 195)
loracarbef	Oral carbacephem; stable in the presence of penicillinases from *Haemophilus* and *Moraxella*
4-Quinolones	
norfloxacin, pefloxacin	Oral quinolones; only in urinary tract infections
ciprofloxacin, ofloxacin, fleroxacin, enoxacin	Oral and systemic quinolones with broad spectrum of indications
levofloxacin, sparfloxacin	Quinolones with enhanced activity against Gram-positive and "atypical" pathogens (chlamydias, mycoplasmas); caution—sparfloxacin is phototoxic
gatifloxacin, moxifloxacin	Quinolones with enhanced activity against Gram-positive and "atypical" pathogens (chlamydias, mycoplasmas) and Gram-negative anaerobes
Cephalosporins	
Group 1	
cefazolin, cephalothin	Effective against Gram-positive and some Gram-negative bacteria; stable in the presence of staphylococci penicillinases; unstable in the presence of betalactamases of Gram-negative bacteria

Table 3.4 *Continued: Frequently Used Anti-Infective Agents*

Class/active substance	Remarks
Group 2 cefuroxime, cefotiam, cefamandole	Effective against Gram-positive bacteria; more effective against Gram-negative bacteria than Group 1; stable in the presence of staphylococci penicillinases; stable in the presence of some betalactamases of Gram-negative bacteria
Group 3a cefotaxime, ceftriaxone, ceftizoxime, cefmenoxime, cefodizime	Much more effective than Group 1 against Gram-negative bacteria; stable in the presence of numerous betalactamases of Gram-negative bacteria; show weak activity against staphylococci
Group 3b Ceftazidime, cefepime, cefpirome, cefoperazone	Spectrum of action as in Group 3a; also effective against *Pseudomonas aeruginosa*
Further cephalosporins cefsulodin	Narrow spectrum of action; the only therapeutically relevant activity is that against *Pseudomonas aeruginosa*
cefoxitin	Effective against the anaerobic *Bacteroidaceae*; activity against Gram-negative bacteria as in Group 2; insufficient activity against staphylococci
Oral cephalosporins ceflaclor, cefadroxil, cephalexin, cefradine	Spectrum of action similar as cephalothin
cefpodoxime, cefuroxime (axetil), cefixime, cefprozil, cefdinir, cefetamet, ceftibuten	Newer oral cephalosporins with broad spectra of action
Chloramphenicol	Broad spectrum, mainly bacteriostatic effect; risk of aplastic anemia
Diaminobenzyl pyrimidine trimethoprim	Broad spectrum; inhibition of dihydrofolic acid reductase; frequent bactericidal synergism with sulfonamides (e.g., cotrimoxazole)

Table 3.4 *Continued: Frequently Used Anti-Infective Agents*

Class/active substance	Remarks
Ethambutol	Only against tuberculosis bacteria
Fosfomycin	Broad spectrum, bactericidal effect in bacterial cell division phase; blocks murein biosynthesis; rapid development of resistance; use in combination therapy
Fusidic acid	Steroid antibiotic; only against Gram-positive bacteria; bacteriostatic; blocks protein biosynthesis (translation); development of resistance is frequent
Glycopeptides vancomycin teicoplanin	Narrow spectrum including only Gram-positive bacteria; moderate bactericidal efficacy during bacterial cell division phase; blocks murein biosynthesis; nephrotoxicity, allergy, thrombophlebitis
Isonicotinamides isoniazid (INH)	Only against tuberculosis bacteria, inhibition of enzymes requiring pyridoxal or pyridoxamine as a coenzyme
Lincosamides lincomycin, clindamycin	Effective against Gram-positive bacteria and Gram-negative anaerobes; good penetration into bone tissue
Macrolides/ketolides	
erythromycin, roxithromycin, clarithromycin, azithromycin	Against Gram-positive and Gram-negative cocci, chlamydias, and mycoplasmas
telithromycin	Ketolide; effective against many macrolide-resistant strains

Table 3.**4** *Continued: Frequently Used Anti-Infective Agents*

Class/active substance	Remarks
Monobactams	Betalactam antibiotics with only the betalactam ring (see fig. 3.**29**, p. 195)
aztreonam, carumonam	Good activity against *Enterobacteriaceae*; moderate efficacy against *Pseudomonas*; very high level of betalactamase stability; no effect against Gram-positive bacteria
Nitrofurans	
nitrofurantoin, furazolidone, nitrofural, etc.	Against Gram-positive and Gram-negative bacteria; use only in urinary tract infections
Nitroimidazoles	
metronidazole, tinidazole, omidazole	Active against various protozoans and obligate anaerobic bacteria; bactericidal effect
Oxalactams	Betalactam antibiotics with oxygen instead of sulfur in the second ring system (see Fig. 3.**29**, p. 195)
lamoxactam	Broad spectrum; moderate efficacy against *Pseudomonas*; poor efficacy against Gram-positive cocci; highly stable in the presence of beta-lactamases; also effective against Gram-negative anaerobes
flomoxef	No activity against *Pseudomonas*; good activity against staphylococci; otherwise like lamoxactam
clavulanic acid	Only minimum antibacterial activity; inhibits beta-lactamases; used in combination with amoxicillin (Augmentin)
Oxazolidinones	
linezolid	Only against Gram-positive bacteria; inhibits bacterial translation; no crossresistance with other translation inhibitors
Para-aminosalicylic acid (PAS)	Only against tuberculosis bacteria; affects folic acid biosynthesis

Table 3.4 *Continued: Frequently Used Anti-Infective Agents*

Class/active substance	Remarks
Penicillins	
Classic penicillins	
penicillin G (benzyl penicillin), penicillin V (oral penicillin), pheneticillin, propicillin	Effective against Gram-positive bacteria and Gram-negative cocci; bactericidal effect during bacterial cell division phase; inactivated by penicillinase of staphylococci, gonococci, *Haemophilus influenzae*, *Moraxella catarrhalis*
Penicillinase-resistant penicillins	
methicillin, oxacillin, cloxacillin, flucloxacillin	Stable in the presence of penicillinase of staphylococci; agent of choice in staphylococci infections (flucloxacillin)
Aminopenicillins	
ampicillin, amoxicillin, epicillin, hetacillin, etc.	Also effective against *Enterobacteriaceae*; labile against Gram-positive and Gram-negative penicillinases
Carboxyl penicillins	
carbenicillin, ticarcillin, carfecillin, etc.	Effective against *Enterobacteriaceae* and *Pseudomonas*; labile against Gram-positive and Gram-negative penicillinases
temocillin (6-α-methoxy ticarcillin)	No effect against *Pseudomonas*; highly stable in the presence of betalactamases
Acylureidopenicillins	
azlocillin, mezlocillin, piperacillin, apalcillin	Effective against *Enterobacteriaceae* and *Pseudomonas*; despite lability against beta-lactamases active against many enzyme-producing strains due to good penetration and high levels of sensitivity of the target molecules
Penems	Penicillins with a double bond in the second ring system
N-formimidoyl thienamycin (imipenem = N-F-thienamycin + cilastatin)	A carbapenem (C atom instead of sulfur in second ring); very broad spectrum and high level of activity against Gram-positive and Gram-negative bacteria, including anaerobes; frequently effective against *Enterobacteriaceae* and *Pseudomonas* with resistance to the cephalosporins of Group 3b; inactivated by renal enzymes; is therefore administered in combination with the enzyme inhibitor cilastatin

Table 3.4 *Continued: Frequently Used Anti-Infective Agents*

Class/active substance	Remarks
meropenem	Like imipenem, but stable against renal dehydropeptidase
Polypeptides	
bacitracin	Only against Gram-positive bacteria; is only used topically
Polymyxin B, colistin	Only against Gram-negative rod bacteria; neurotoxicity, nephrotoxicity
Rifamycins	
rifampicin	Against Gram-positive bacteria and tuberculosis bacteria; mainly bacteriostatic; rapid development of resistance, for which reason combination therapy is recommended
Streptogramins	
quinopristin/dalfopristin	Fixed combination preparation of two streptogramins; effective mainly against Gram-positive bacteria
Sulfamethoxazole/trimethoprim (cotrimoxazole)	Fixed combination; five parts sulfamethoxazole and one part trimethoprim
Sulfonamides	
sulfanilamide, sulfamethoxazole, sulfafurazole, etc.	Broad spectrum; bacteriostatic effect only; resistance frequent
Sulfones	
dapsone	diaminodiphenylsulfone; for therapy of leprosy
Tetracyclines	
doxycycline tetracycline, oxytetracycline, rolitetracycline, minocycline	Broad spectrum including all bacteria, chlamydias, and rickettsias; resistance frequent; dental deposits in small children

Basic Chemical Structures of the Most Important Anti-infectives

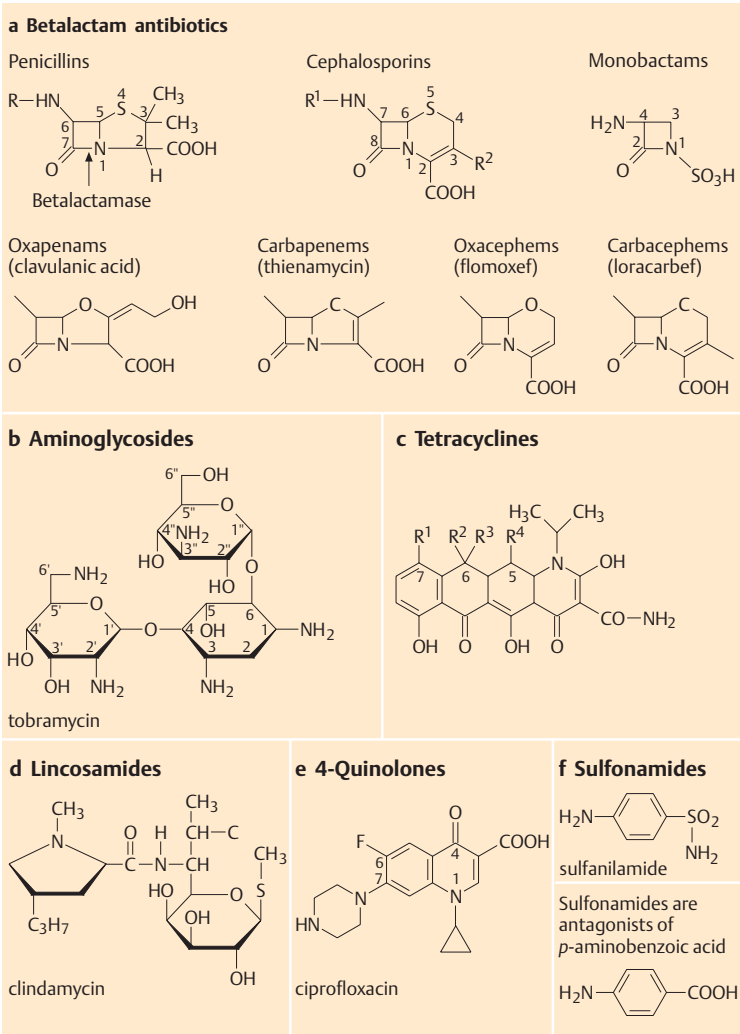

Fig. 3.**29** Antibiotic groups often include many drug substances with different substituents.

Spectrum of Action

Each anti-infective agent has a certain **spectrum of action**, which is a range of bacterial species showing natural sensitivity to the substance. Some anti-infective agents have a narrow spectrum of action (e.g., vancomycin). Most, however, have broad spectra like tetracyclines, which affect all eubacteria.

Efficacy

The efficacy of an anti-infective agent (syn. kinetics of action) defines the way it affects a bacterial population. Two basic effects are differentiated: **bacteriostasis**, i.e., reversible inhibition of growth, and irreversible **bactericidal activity** (Fig. 3.**30**). Many substances can develop both forms of efficacy depending on their concentration, the type of organism, and the growth phase. Many of these drugs also have a **postantibiotic effect (PAE)** reflecting the damage inflicted on a bacterial population. After the anti-infective agent is no longer present, the bacterial cells not killed require a recovery phase before they can reproduce again. The PAE may last several hours.

A bacteriostatic agent alone can never completely eliminate pathogenic bacteria from the body's tissues. "Healing" results from the combined effects of the anti-infective agent and the specific and nonspecific immune defenses of the host organism. In tissues in which this defense system is inefficient (endocardium), in the middle of a purulent lesion where no functional phagocytes are present, or in immunocompromised patients, bactericidal substances must be required. The clinical value of knowing whether an antibacterial drug is bacteriostatic or bactericidal is readily apparent.

All of the bacteria from an infection focus cannot be eliminated without support from the body's immune defense system. A bacterial population always includes several cells with phenotypic resistance that is not genotypically founded. These are the so-called **persisters**, which occur in in-vitro cultures at frequencies ranging from $1{:}10^6$ to $1{:}10^8$ (Fig. 3.**30**). The cause of such persistence is usually a specific metabolic property of these bacteria that prevents bactericidal substances from killing them. Following discontinuation of therapy, such persisters can lead to relapses. Infections with **L-forms** show a special type of persistence when treated with antibiotics that block murein synthesis (p. 156).

Efficacy of Selected Anti-Infective Agents

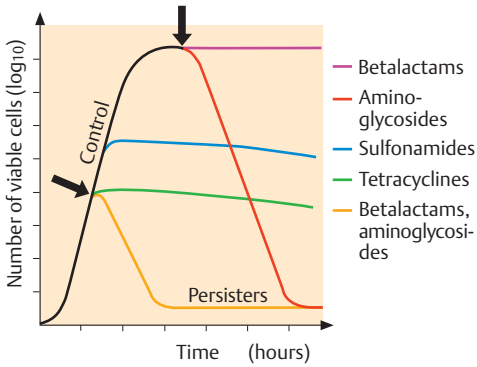

Fig. 3.**30** The arrows indicate addition of substances in the different phases of the normal growth curve (see Fig. 3.**16**). Betalactams are bactericidal only during the bacterial cell division phase, whereas aminoglycosides show this activity in all growth phases. Sulfonamides are always bacteriostatic, tetracyclines are mainly bacteriostatic. Some cells in every culture (so-called persisters) are phenotypically (but not genotypically) resistant to the bactericidal effects of anti-infective agents.

Mechanisms of Action

Table 3.**5** provides a concise summary of the molecular mechanisms of action of the most important groups of anti-infective agents.

Table 3.**5** Mechanisms of Action of the Most Important Anti-Infective Agents

Substance group	Mechanism, activity site
Sulfonamides	Competition with *p*-aminobenzoic acid as a substrate for dihydropteric acid synthetase, thus too little tetrahydrofolic acid
Trimethoprim	Inhibition of dihydrofolic acid reductase, thus too little tetrahydrofolic acid
Betalactam antibiotics	Disturbance of murein biosynthesis: — Irreversible inhibition of DD-transpeptidase, which catalyzes the peptide crosslinkage in murein — Release of an inhibitor of autolytic murein enzymes — Enzymatic destruction of murein architecture with autolysins: "wrong place at the wrong time" — Lysis due to high internal osmotic pressure
Vancomycin Teicoplanin Fosfomycin Bacitracin	Disturbance of murein biosynthesis at various different molecular stages
Rifamycin	Transcription: Blockage of DNA-dependent RNA polymerase
Aminoglycosides	Translation: — Genetic code is not read correctly (miscoding) — Blockage of e-type (elongation ribosome) A-position occupancy by AA-tRNA
Tetracyclines	Translation: — Blockage of e-type (elongation ribosome) and i-type (initiation ribosome) A-position occupancy by AA-tRNA
Chloramphenicol	Translation: Inhibition of peptidyl transferase activity
Macrolides, ketolides	Translation: Inhibition of elongation of the polypeptide chain
4-Quinolones	Inhibition of the DNA gyrase and topoisomerase IV resulting in the inhibition of DNA replication
Polymyxins	Cytoplasmic membrane: Structural disruption

Details of the Mechanisms of Action of Anti-Infective Agents

Sulfonamides and trimethoprim	Tetrahydrofolic acid (THFA) acts as a coenzyme to regulate the C1 metabolism for transfer of the hydroxymethyl and formyl groups. Too little THFA results in the cessation of growth. The combination of sulfamethoxazole and trimethoprim (cotrimoxazole) results in a potentiated efficacy.
Betalactam antibiotics	The mechanisms described in Table 3.**5** refer to penicillin and pneumococci. They probably hold in similar form for other betalactams and other bacteria as well. All bacteria with cell walls containing murein possess autolysins. These enzymes create gaps in the murein sacculus while the bacterium is growing, these gaps are then filled in with new murein material. Bacteria the growth of which is inhibited, but which are not lysed, show betalactam **tolerance** (bacteriostatic, but not bactericidal effects).
Protein synthesis inhibitors	The biosynthesis of bacterial proteins differs in detail from that observed in eukaryotes, permitting a selective inhibition by antibiotics. The principle of selective toxicity still applies.
4-Quinolones	DNA gyrase, which only occurs in bacteria, catalyzes the counterclockwise supercoiling of the double helix, which is, in itself, wound to the right, about its helical axis (see Fig. 3.**17**, p. 167). Only in supercoiled form can the DNA fit economically into the cell. DNA replication depends on this supercoiled topology. 4-Quinolones also inhibit bacterial topoisomerase IV of Gram-positive bacteria.

Pharmacokinetics

Pharmacokinetics covers the principles of absorption, distribution, and elimination of pharmacons by the macroorganism. The reader is referred to standard textbooks of pharmacology for details. The dosage and dosage interval recommendations for antibacterial therapy take into account the widely differing pharmacokinetic parameters of the different anti-infective agents, among them:

- Absorption rate and specific absorption time
- Volume of distribution
- Protein binding
- Serum (blood) concentration
- Tissue concentration
- Metabolization
- Elimination

Side Effects

Treatment with anti-infective agents can cause side effects, resulting either from noncompliance with important therapeutic principles or specific patient reactivity. On the whole, such side effects are of minor significance.

- **Toxic effects.** These effects arise from direct cell and tissue damage in the macroorganism. Blood concentrations of some substances must therefore be monitored during therapy if there is a risk of cumulation due to inefficient elimination (examples: aminoglycosides, vancomycin).

- **Allergic reactions.** See p. 108 for possible mechanisms (example: penicillin allergy).

- **Biological side effects.** Example: change in or elimination of normal flora, interfering with its function as a beneficial colonizer (see p. 25).

The Problem of Resistance

Definitions

Clinical resistance. Resistance of bacteria to the concentration of anti-infective agents maintained at the infection site in the macroorganism.

Natural resistance. Resistance characteristic of a bacterial species, genus, or family.

Acquired resistance. Strains of sensitive taxa can acquire resistance by way of changes in their genetic material.

Biochemical resistance. A biochemically detectable resistance observed in strains of sensitive taxa. The biochemical resistance often corresponds to the clinically relevant resistance. Biochemically resistant strains sometimes show low levels of resistance below the clinically defined boundary separating resistant and sensitive strains. Such strains may be medically susceptible.

Incidence, Significance

Problematic bacteria. Strains with acquired resistance are encountered frequently among *Enterobacteriaceae*, pseudomonads, staphylococci, and enterococci. Specific infection therapy directed at these pathogens is often fraught with difficulties, which explains the label problematic bacteria. They are responsible for most nosocomial infections (p. 342f.). Usually harmless in otherwise healthy persons, they may cause life-threatening infections in highly susceptible, so-called **problematic patients**. Problematic bacteria are often characterized by **multiple resistance**. Resistance to anti-infective agents is observed less frequently in nonhospital bacteria.

Genetic variability. The basic cause of the high incidence of antibiotic resistance experienced with problematic bacteria is the pronounced genetic variability of these organisms, the mechanisms of which are described in the section “Genetic variability” (p. 171 and p. 174). Most important are the mechanisms of horizontal transfer of resistance determinants responsible for the efficient distribution of resistance markers among these bacteria.

Selection. The origin and distribution of resistant strains is based to a significant extent on selection of resistance variants. The more often anti-infective substances are administered therapeutically, the greater the number of strains that will develop acquired resistance. Each hospital has a characteristic flora reflecting its prescription practice. A physician must be familiar with the resistance characteristics of this hospital flora so that the right

anti-infective agents for a **"calculated antibiotic therapy"** can be selected even before the resistance test results are in. Such therapies take into account the frequency of infections by certain bacterial species (pathogen epidemiology) as well as current resistance levels among these bacteria (resistance epidemiology).

Resistance Mechanisms

Inactivating enzymes. Hydrolysis or modification of anti-infective agents.

- **Betalactamases.** Hydrolyze the betalactam ring of betalactam antibiotics (see Fig. 3.**29**). Over 200 different betalactamases are known. A course classification system is based on the substrate profile in penicillinases and cephalosporinases. Production of some betalactamases is induced by betalactams (see p. 169), others are produced constitutively (unregulated).

- **Aminoglycosidases.** Modify aminoglycosides by means of phosphorylation and nucleotidylation of free hydroxyl groups (phosphotransferases and nucleotidyl transferases) or acetylation of free amino groups (acetyltransferases).

- **Chloramphenicol acetyltransferases.** Modification, by acetylation, of chloramphenicol.

Resistant target molecules.

- Gene products with a low affinity to anti-infective agents are produced based on mutations in natural genes. Example: DNA gyrase subunit A, resistant to 4-quinolones.

- Acquisition of a gene that codes for a target molecule with low affinity to anti-infective agents. The resistance protein assumes the function of the sensitive target molecule. Example: methicillin resistance in staphylococci; acquisition of the penicillin-binding protein 2a, which is resistant to betalactam antibiotics and assumes the function of the naturally sensitive penicillin-binding proteins.

- Acquisition of the gene for an enzyme that alters the target structure of an anti-infective agent to render it resistant. Example: 23S rRNA methylases; modification of ribosomal RNA to prevent binding of macrolide antibiotics to the ribosome.

Permeability mechanisms.

- **Reduced influx.** Reduction of transport of anti-infective agents from outside to inside through membranes; rare.

Increased efflux. Active transport of anti-infective agents from inside to outside by means of efflux pumps in the cytoplasmic membrane, making efflux greater than influx; frequent.

Evolution of Resistance to Anti-Infective Agents

Resistance to anti-infective agents is genetically determined by resistance genes. Many resistance determinants are not new developments in response to the use of medical antibiotics, but developed millions of years ago in bacteria with no human associations. The evolutionary process is therefore a **"nonanthropogenic"** one. The determinants that code for resistance to anti-infective agents that are not antibiotics did develop after the substances began to be used in therapy, hence this is **"anthropogenic"** evolution. Factors contributing to the resistance problem have included the molecular mechanisms of genetic variability (mutation, homologous recombination, site-specific integration, transposition) and the mechanisms of intercellular gene transfer in bacteria (transformation, transduction, conjugation).

Nonanthropogenic and Anthropogenic Evolution

Nonanthropogenic evolution. The need for resistance developed parallel to the ability to produce antibiotics. The producing organisms protect themselves from their own products by means of such R mechanisms. Resistance genes also evolved in bacteria that shared the natural habitat of the antibiotic producers. They secured their own ecological niche in the presence of the producers by means of the characteristic of resistance. The genetic sequences from which the resistance genes evolved were those that coded for the anabolic or catabolic metabolism genes. At a later point in evolutionary history, such "nonanthropogenic" genes have accidentally, and rarely, found their way into the genetic material of human pathogen bacteria. Therefore, when new antibiotic substances come to be used for therapeutic purposes, there are always a small number of bacteria that already show resistance to them.

Anthropogenic evolution. This term refers to the evolution of resistance genes in bacteria associated with humans based on mutations in native genes. An example is the mutation that brings about resistance to 4-quinolones in gene *gyrA*, which codes for subunit A of the DNA gyrase. A special case of anthropogenic evolution is the development of new resistance genes resulting from mutations in "nonanthropogenic" resistance genes already established in human pathogen bacteria.

The best-known example of this is provided by mutations in TEM and SHV *betalactamase* genes that code for betalactamases with a very broad substrate profile (ESBL = extended spectrum betalactamases).

Resistance Tests

Two standard test systems are used to determine the in-vitro resistance levels of bacteria.

In **dilution series tests**, the minimum inhibitory concentration (MIC) of an anti-infective agent required to inhibit proliferation of a bacterial population is determined. A factor 2 geometrical dilution series of the agent is prepared in a nutrient medium, inoculated with the test organism and incubated, whereupon the lowest growth-inhibiting concentration level (mg/l) is determined. Three standardized dilution methods are available. In the agar dilution test, nutrient agar plates containing antibiotic are inoculated ("spotted") with the test organisms. In the microbroth dilution test, the final volume is usually 100 µl per microplate well. This test type can also be automated. The final volume in a macrobroth dilution test is 2 ml per tube.

Due to the complexity and time-consuming nature of the above test types, routine laboratories often use the **agar diffusion test**. This involves diffuse inoculation of the nutrient agar plate with the test strain. Then disks of filter paper containing the anti-infective agents are placed on the agar. After the plates thus prepared are incubated, the inhibition zones around the disks (i.e., whether or not they develop and their size) provide information on the resistance of the microorganisms tested (Fig. 3.**31**). This is possible because of the linear relation between the $\log_2$ MIC and the diameter of the inhibition zones (Fig. 3.**32**).

To **interpret the results**, the MICs or inhibition zones are brought into relation with the substance concentrations present at a site of infection at standard dosage levels. This calculation is based on known averages for various pharmacokinetic parameters (serum concentration, half-life) and pharmacodynamic parameters (bactericidal activity or not, postantibiotic effect,

Agar Diffusion Test

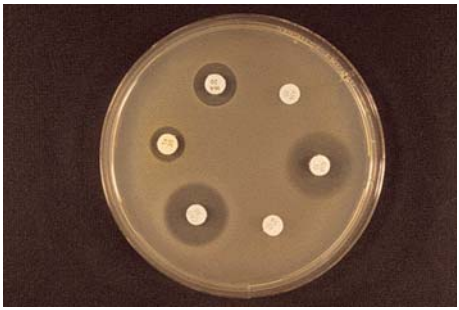

Fig. 3.**31** This method, also known as the "disk test," is used to test the resistance of a bacterial culture to various anti-infective agents. The method provides a basis for classification of a bacterial strain as "susceptible," "resistant," or "intermediate" according to the dimension of the inhibition zone.

Inhibition Zone and Minimum Inhibitory Concentration (MIC)

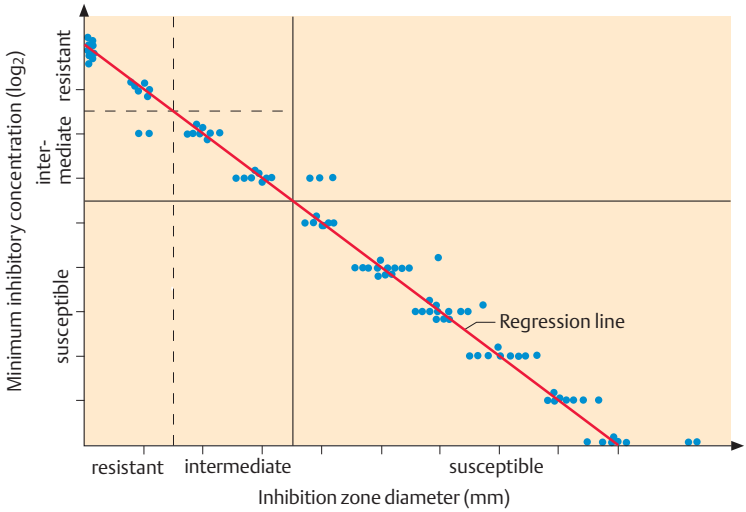

Fig. 3.**32** Each point represents a bacterial strain. The size of the inhibition zone is determined in the agar diffusion test (disk test) and the minimum inhibitory concentration (MIC) in the dilution test. The MIC corresponds logarithmically (log_2) with the diameter of the inhibition zone.

etc.). The interpretation also takes into account clinical experience gained from therapy of infections with pathogens of given suceptibility. Such data are used to establish general guideline values defining the boundary between susceptible and resistant bacteria.

The **minimum bactericidal concentration** (MBC) is the smallest concentration of a substance required to kill 99.9% of the cells in an inoculum.

The MBC is determined using quantitative subcultures from the macroscopically unclouded tubes or (microplate) wells of an MIC dilution series.

Combination Therapy

Combination therapy is the term for concurrent administration of two or more anti-infective agents. Some galenic preparations combine two components in a fixed ratio (example: cotrimoxazole). Normally, however, the in-

dividual substances in a combination therapy are administered separately. Several different objectives can be pursued with combination therapy:

- **Broadening of the spectrum of action.** In mixed infections with pathogens of varying resistance; in calculated therapy of infections with unknown, or not yet known, pathogenic flora and resistance characteristics.
- **Delay of resistance development.** In therapy of tuberculosis; when using anti-infective agents against which bacteria quickly develop resistance.
- **Potentiation of efficacy.** In severe infections requiring bactericidal activity at the site of infection. Best-known example: penicillin plus gentamicin in treatment of endocarditis caused by enterococci or streptococci.

Combining the effects of anti-infective drugs can have several different effects:

- **No difference.** The combination is no more efficacious than the more active of the two components alone.
- **Addition.** Summation of the effects.
- **Synergism.** Potentiation of the effects.
- **Antagonism.** The combination is less efficacious than one of the two components alone.

Rule of thumb: combinations of bacteriostatics with substances that are bactericidal in the cell division phase only often result in antagonism, e.g., penicillin plus tetracycline in therapy of pneumococcal pneumonia.

In-vitro investigations of the mechanism of action of a combination when used against a pathogen usually employ the so-called "checkerboard titration" technique, in which the combinatory effects of substances A and B are compared using a checkerboard-like pattern.

Chemoprophylaxis

One of the most controversial antibiotic uses is prophylactic antibiosis. There are no clear-cut solutions here. There are certain situations in which chemoprophylaxis is clearly indicated and others in which it is clearly contraindicated. The matter must be decided on a case-by-case basis by weighing potential benefits against potential harm (side effects, superinfections with highly virulent and resistant pathogens, selection of resistant bacteria).

Chemoprophylaxis is considered useful in malaria, rheumatic fever, pulmonary cystic fibrosis, recurring pyelonephritis, following intensive contact

with meningococci carriers, before surgery involving massive bacterial contamination, in heavily immunocompromised patients, in cardiac surgery or in femoral amputations due to circulatory problems. Chemoprophylaxis aimed at preventing a postsurgical infection should begin a few hours before the operation and never be continued for longer than 24–72 hours.

Immunomodulators

Despite the generally good efficacy of anti-infective agents, therapeutic success cannot be guaranteed. Complete elimination of bacterial pathogens also requires a functioning immune defense system. In view of the fact that the number of patients with severe immunodeficiencies is on the rise, immunomodulators are used as a supportive adjunct to specific antibiotic therapy in such patients. Many of these "cytokines" (see p. 77ff.) produced by the cells of the immune system can now be produced as "recombinant proteins." Myelopoietic growth factors have now been successfully used in patients suffering from neutropenia. Additional immunomodulators are also available, e.g., interferon gamma (IFNγ) and interleukin 2 (IL-2).

Laboratory Diagnosis

Infections can be diagnosed either directly by detection of the pathogen or components thereof or indirectly by antibody detection methods. The reliability of laboratory results is characterized by the terms sensitivity and specificity, their value is measured in terms of positive to negative predictive value. These predictive values depend to a great extent on prevalence. In direct laboratory diagnosis, correct material sampling and adequate transport precautions are an absolute necessity. The classic methods of direct laboratory diagnosis include microscopy and culturing. Identification of pathogens is based on morphological, physiological, and chemical characteristics. Among the latter, **the importance of detection of pathogen-specific nucleotide sequences is constantly increasing**. Development of sensitive test systems has made direct detection of pathogen components in test materials possible in some cases. The molecular biological methods used are applied with or without amplification of the sequence sought as the case warrants. Direct detection can also employ polyclonal or monoclonal antibodies to detect and identify antigens.

Preconditions, General Methods, Evaluation

Preconditions

The field of medical microbiology dealing with laboratory diagnosis of infectious diseases is known as diagnostic or clinical microbiology. Modern medical practice, and in particular hospital-based practice, is inconceivable without the cooperation of a special microbiological laboratory.

To ensure optimum patient benefit, the physician in charge of treatment and the laboratory staff must cooperate closely and efficiently. The preconditions include a basic knowledge of pathophysiology and clinical infectiology on the part of the laboratory staff and familiarity with the laboratory work on the part of the treating physician. The following sections provide a brief rundown on what physicians need to know about laboratory procedures.

General Methods and Evaluation

An infectious disease can be diagnosed **directly** by finding the causal pathogen or its components or products. It can also be diagnosed **indirectly** by means of antibody detection (Chapter 2, p. 121ff.). The accuracy and value of each of the available diagnostic methods are characterized in terms of sensitivity, specificity, and positive or negative predictive value. These parameters are best understood by reference to a 2 × 2 table (Table 3.**6**).

By inserting fictitious numbers into the 2 × 2 table, it readily becomes apparent that a positive predictive value will fall rapidly, despite high levels of specificity and sensitivity, if the prevalence level is low (Baye's theorem).

Sampling and Transport of Test Material

It is very important that the material to be tested be correctly obtained (sampled) and transported. In general, material from which the pathogen is to be isolated should be sampled as early as possible before chemotherapy is begun. Transport to the laboratory must be carried out in special containers provided by the institutes involved, usually containing transport mediums—either enrichment mediums (e.g., blood culture bottle), selective growth mediums or simple transport mediums without nutrients. An invoice must be attached to the material containing the information required for processing (using the form provided).

Table 3.6 2 × 2 Table: Explanation and Calculation of Sensitivity, Specificity, and Predictive Value (Positive–Negative)

Collective	Test positive	Test negative
Infected	Correct positive *cp*	False negative *fn*
Noninfected	False positive *fp*	Correct negative *cn*

- Sensitivity (%) measures the frequency of correct positive results in the **infected** collective (horizontal addition).
- Specificity (%) measures the frequency of correct negative results in the **noninfected** collective (horizontal addition).

$$\text{Sensitivity (\%)} = \frac{cp}{cp + fn} \times 100; \quad \text{Specificity (\%)} = \frac{cn}{fp + cn} \times 100$$

- The predictive value of the positive result expresses the probability that a positive result indicates an infection. It analyzes the positive test results both in the infected collective and in the noninfected collective (vertical addition).
- The predictive value of the negative result expresses the probability that a negative result indicates noninfection.

$$\text{Pos. pred. value (\%)} = \frac{cp}{cp + fp} \times 100; \quad \text{Neg. pred. value (\%)} = \frac{cn}{cn + fn} \times 100$$

Material from the respiratory tract:

- Swab smear from tonsils.
- Sinus flushing fluid.
- Pulmonary secretion. Expectorated sputum is usually contaminated with saliva and the flora of the oropharynx. Since these contaminations include pathogens that may cause infections of the lower respiratory tract organs, the value of positive findings would be limited. The material can be considered unsuitable for diagnostic testing if more than 25 oral epithelia are present per viewing frame at 100× magnification. Morning sputum from flushing the mouth or after induction will result in suitable samples. Sputum is not analyzed for anaerobes.
- Useful alternatives to expectorated sputum include bronchoscopically sampled bronchial secretion, flushing fluid from bronchoalveolar lavage (BAL), transtracheal aspirate or a pulmonary puncture biopsy. These types of material are required if an anaerobe infection is suspected. The material must then be transported in special anaerobe transport containers.

Material from the urogenital tract:

- Urine. Midstream urine is in most cases contaminated with the flora of the anterior urethra, which often corresponds to the pathogen spectrum of urinary tract infections. Bacterial counts must be determined if "contamination" is to be effectively differentiated from "infection." At counts in morning urine of $\geq 10^5$/ml an infection is highly probable, at counts of $\leq 10^3$ rather improbable. At counts of around 10^4/ml the test should be repeated. Lower counts may also be diagnostically significant in urethrocystitis. The dipstick method, which can be used in any medical practice, is a simple way of estimating the bacterial count: a stick coated with nutrient medium is immersed in the midstream urine, then incubated. The colony count is then estimated by comparing the result with standardized images.
- Catheterizing the urinary bladder solely for diagnostic purposes is inadvisable due to the potential for iatrogenic infection. Uncontaminated bladder urine is obtainable only by means of a suprapubic bladder puncture.
- Genital secretions are sampled with smear swabs and must be transported in special transport mediums.

Blood:

- For a blood culture, at least 10–20 ml of venous blood should be drawn sterilely into one aerobic and one anaerobic blood culture bottle. Sample three times a day at intervals of several hours (minimum interval one hour).
- For serology, (2–)5 ml of native blood will usually suffice. Take the initial sample as early as possible and a second one 1–3 weeks later to register any change in the antibody titer.

Pus and wound secretions:

- For surface wounds sample material with smear swabs and transport in preservative transport mediums. Such material is only analyzed for aerobic bacteria.
- For deep and closed wounds, liquid material (e.g., pus) should be sampled, if possible, with a syringe. Use special transport mediums for anaerobes.

Material from the gastrointestinal tract:

- Use a small spatula to place a portion of stool about the size of a cherry in liquid transport medium for shipment.
- Transport duodenal juice and bile in sterile tubes. Use special containers if anaerobes are suspected.

Cerebrospinal fluid, puncture biopsies, exudates, transudates:

- Ensure sampling sterility. Use special containers if anaerobes are suspected.

Microscopy

Bacteria are so small that a magnification of 1000× is required to view them properly, which is at the limit of light microscope capability. At this magnification, bacteria can only be discerned in a preparation in which their density is at least 10^4–10^5 bacteria per ml.

Microscopic examination of such material requires a slide preparation:

- **Native preparations**, with or without vital staining, are used to observe living bacteria. The poor contrast of such preparations makes it necessary to amplify this aspect (dark field and phase contrast microscopy). Native preparations include the coverslip and suspended drop types.

- **Stained preparations** are richer in contrast so that bacteria are readily recognized in an illuminated field at 1000×. The staining procedure kills the bacteria. The material is first applied to a slide in a thin layer, dried in the air, and fixed with heat or methyl alcohol. **Simple** and **differential staining techniques** are used. The best-known simple staining technique employs methylene blue. **Gram staining** is the most important differential technique (Table 3.**7**): Gram-positive bacteria stain blue-violet, Gram-negative bacteria stain red. The Gram-positive cell wall prevents alcohol elution of the stain-

Table 3.**7** Procedure for the Three Most Important Types of Staining

Methylene blue	Gram staining	Ziehl–Neelsen staining
Methylene blue 1–5 minutes	Gentian violet or crystal violet, 1 minute	Concentrated carbolfuchsin; heat three times until vapor is observed
Rinse off with water	Pour off stain, rinse off with Lugol's solution, then cover with Lugol's solution for 2–3 minutes	Rinse off with water Destain with HCl (3%)/alcohol mixture
	Pour off Lugol's solution	Counterstain with methylene blue, 1–5 minutes
	Destain with acetone/ethyl alcohol (1:4)	Rinse off with water
	Rinse off with water	
	Counterstain with dilute carbolfuchsin, 1 minute	
	Rinse off with water	

iodine complex. In old cultures in which autolytic enzymes have begun to break down the cell walls, Gram-positive cells may test Gram-negative ("Gram-labile" bacteria).

Another differential stain is the **Ziehl-Neelsen** technique. It is used to stain mycobacteria, which do not "take" gram or methylene blue stains due to the amounts of lipids in their cell walls. Since mycobacteria cannot be destained with HCl-alcohol, they are called acid-resistant rods. The mycobacteria are stained red and everything else blue.

Fluorescence microscopy is another special technique. A fluorochrome absorbs shortwave light and emits light with a longer wavelength. Preparations stained with fluorochromes are exposed to light at the required wavelength. The stained particles appear clearly against a dark background in the color of the emitted light. This technique requires special equipment. Its practical application is in the observation of mycobacteria. In **immunofluorescence** detection, a fluorochrome (e.g., fluorescein isothiocyanate) is coupled to an antibody to reveal the presence of antigens on particle surfaces.

Culturing Methods

Types of nutrient mediums. Culturing is required in most cases to detect and identify bacteria. Almost all human pathogen bacteria can be cultivated on nutrient mediums. Nutrient mediums are either liquid (nutrient broth) or gelatinous (nutrient agar, containing 1.5–2% of the polysaccharide agarose). Enrichment mediums are complex mediums that encourage the proliferation of many different bacterial species. The most frequently used enrichment medium is the blood agar plate containing 5% whole blood. Selective mediums allow only certain bacteria to grow and suppress the reproduction of others. Indicator mediums are used to register metabolic processes.

Proliferation forms. Most bacteria show diffuse proliferation in **liquid mediums**. Some proliferate in "crumbs," other form a grainy bottom sediment, yet others a biofilm skin at the surface (pseudomonads). Isolated colonies are observed to form on, or in, **nutrient agar** if the cell density is not too high. These are pure cultures, since each colony arises from a single bacterium or colony-forming unit (CFU). The pure culture technique is the basis of bacteriological culturing methods. The procedure most frequently used to **obtain isolated colonies** is fractionated inoculation of a nutrient agar plate (Figs. 3.**33**–3.**35**).

Use of this technique ensures that isolated colonies will be present in one of the three sectors. Besides obtaining pure cultures, the isolated colony technique has the further advantage of showing the form, appearance, and

Fractionated Inoculation of Nutrient Agar

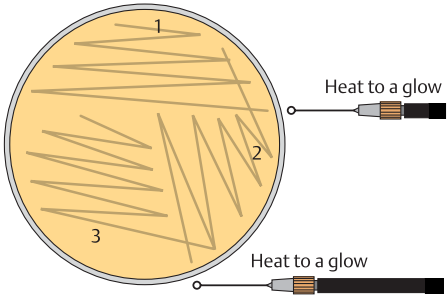

Fig. 3.**33** Isolated colonies are obtained by means of fractionated inoculation of nutrient agar. The wire loop must be sterilized between inoculations.

Blood Agar Plate Following Fractionated Inoculation and Incubation

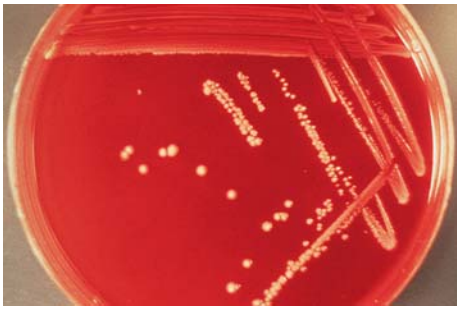

Fig. 3.**34** Blood agar is frequently used as a universal enrichment medium. Most human bacterial pathogens grow on it. Here is a pure culture of *Staphylococcus aureus*.

Endo Agar Following Fractionated Inoculation with a Mixed Culture

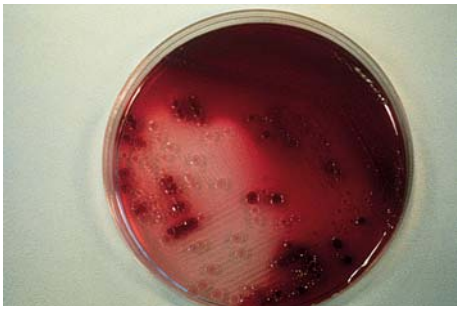

Fig. 3.**35** Endo agar is a combined selective/indicator medium. It allows growth of *Enterobacteriaceae*, *Pseudomonadaceae*, and other Gram-negative rod bacteria but inhibits the growth of Gram-positive bacteria and Gram-negative cocci. The red color of the colonies and agar is characteristic of lactose breakdown (= *Escherichia coli*); the light-colored colonies are lactose-negative (= *Salmonella enterica*).

color of single colonies. The special proliferation forms observed in nutrient broth and nutrient agar give an experienced bacteriologist sufficient information for an initial classification of the pathogen so that identifying reactions can then be tested with some degree of specificity.

Conditions required for growth. The optimum proliferation temperature for most human pathogen bacteria is 37 °C.

Bacteria are generally cultured under atmospheric conditions. It often proves necessary to incubate the cultures in 5% CO_2. Obligate anaerobes must be cultured in a milieu with a low redox potential. This can be achieved by adding suitable reduction agents to the nutrient broth or by proliferating the cultures under a gas atmosphere from which most of the oxygen has been removed by physical, chemical, or biological means.

Identification of Bacteria

The essential principle of bacterial identification is to assign an unknown culture to its place within the taxonomic classification system based on as few characteristics as possible and as many as necessary (Table 3.**8**).

- **Morphological characteristics**, including staining, are determined under the microscope.
- **Physiological characteristics** are determined with indicator mediums. Commercially available miniaturized systems are now frequently used for this purpose (Fig. 3.**36**).

Determination of Metabolic Characteristics with Indicator Mediums

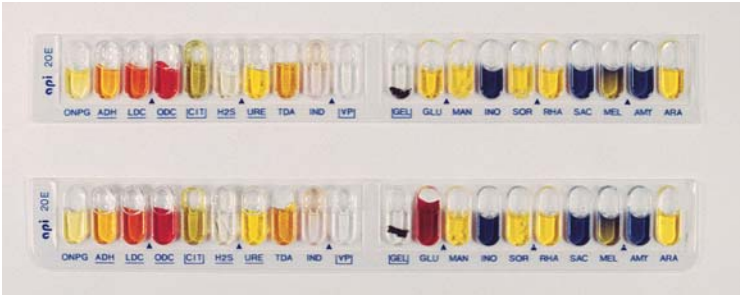

Fig. 3.**36** Identification of *Enterobacteriaceae* using API 20E, a standardized microplate method. Positive and negative reactions are shown by color reactions.

Table 3.**8** Characteristics Useful in Identification of Bacteria

Morphological characteristics
Form (sphere, rod, spiral)
Size; pseudogroupings (clusters, chains, diplococci)
Staining (Gram-positive, Gram-negative); flagella (presence, arrangement); capsule (yes, no); spores (form, within cell formation)
Physiological characteristics
Respiratory chain enzymes (oxidases, catalases)
Enzymes that break down carbohydrates, alcohols, glycosides (e.g., betagalactosidase)
Protein metabolism enzymes (e.g., gelatinase, collagenase)
Amino acid metabolism enzymes (e.g., decarboxylases, deaminases, urease)
Other enzymes: hemolysins, lipases, lecithinases, DNases, etc.
End products of metabolism (e.g., organic acids detected by gas chromatography)
Resistance/sensitivity to chemical noxae
Characteristics of anabolic metabolism (e.g., citrate as sole source of C)
Chemical characteristics
DNA structure (base sequences)
Structure of cell wall murein
Antigen structure: fine structures detectable with antibodies (e.g., flagellar protein or polysaccharides of the cell wall or capsule)
Fatty acids in membranes and cell wall; analysis using different chromatographic methods

■ **Chemical characteristics** have long been in use to identify bacteria, e.g., in detection of antigen structures. Molecular genetic methods (see below) will play an increasing role in the future.

Molecular Methods

The main objective of the molecular methods of bacterial identification is direct recognition of pathogen-specific nucleotide sequences in the test material. These methods are used in particular in the search for bacteria that are not culturable, are very difficult to culture, or proliferate very slowly. Of course, they can also be used to identify pure bacterial cultures (see above). In principle, any species-specific sequence can be used for identification, but the specific regions of genes coding for 16S rRNA and 23S rRNA are particularly useful in this respect. The following methods are used:

DNA probes. Since DNA is made up of two complementary strands of nucleic acids, it is possible to detect single-strand sequences with the hybridization technique using complementary marking of single strands. The probes can be marked with radioactivity (^{32}P, ^{35}S) or nonradioactive reporter molecules (biotin, dioxigenin):

- *Solid phase hybridization*. The reporter molecule or probe is fixed to a nylon or nitrocellulose membrane (colony blot technique, dot blot technique).
- *Liquid phase hybridization*. The reporter molecule and probe are in a solute state.
- *In-situ hybridization*. Detection of bacterial DNA in infected tissue.

Amplification. The main objective here is to increase the sensitivity level so as to find the "needle in a haystack." A number of techniques have been developed to date, which can be classified in three groups:

- *Amplification of the target sequence*. The oldest and most important among the techniques in this group is the polymerase chain reaction (PCR), which is described on p. 409f.). With "real time PCR," a variant of PCR, the analysis can be completed in 10 minutes.
- *Probe amplification*.
- *Signal amplification*.

Identification by Means of Amplification and Sequencing

The target sequence for identification of bacteria that have not yet been cultured (e.g., *Tropheryma whipplei*, the causal pathogen in Whipple's disease) or of pathogens very difficult to identify with classic methods, is often a certain region of the 16S rRNA, some sections of which are identical in all bacteria. Between these highly conservative segments are other sections that are specific for a species or genus. Using primers that can recognize the conserved regions of 16S rDNA to the right and left of the specific regions, the specific sequence is amplified, then sequenced. The base sequence thus obtained is then identified by comparison with a reference data library.

Identification by Means of Amplification and Gene Chips

In this technique, thousands of oligonucleotides specific for human pathogen bacteria are deposited on the surface of a chip about 2 cm^2 in size. This chip is then charged with amplified and marked single-strand DNA from the test material (containing, for example, species-specific sequences of the 16S rDNA or other species-specific sequences). Then the level of binding to complementary nucleotide sequences is measured as fluorescence using confocal laser scanning microscopy. The occurrence of antibiotic resistance genes can also be measured by this method.

Direct Detection of Bacterial Antigens

Antigens specific for particular species or genera can be detected directly by means of polyclonal or (better yet) monoclonal antibodies present in the test material. This allows for rapid diagnosis. Examples include the detection of bacterial antigens in cerebrospinal fluid in cases of acute purulent meningitis, detection of gonococcal antigens in secretion from the urogenital tract, and detection of group A streptococcal antigen in throat smear material. These direct methods are not, however, as sensitive as the classic culturing methods. Adsorbance, coagglutination, and latex agglutination tests are frequently used in direct detection. In the agglutination methods, the antibodies with the Fc components are fixed either to killed staphylococcal protein A or to latex particles.

Diagnostic Animal Tests

Animal testing is practically a thing of the past in diagnostic bacteriology. Until a few years ago, bacterial toxins (e.g., diphtheria toxin, tetanus toxin, botulinus toxin) were confirmed in animal tests. Today, molecular genetic methods are used to detect the presence of the toxin gene, which process usually involves an amplification step.

Bacteriological Laboratory Safety

Microbiologists doing diagnostic work will of course have to handle potentially pathogenic microorganisms and must observe stringent regulations to avoid risks to themselves and others. Laboratory safety begins with suitable room designs and equipment (negative-pressure lab rooms, safety hoods)

and goes on to include compliance with the basic rules of work in a microbiological laboratory: protective clothing, no eating, drinking, or smoking, mechanical pipetting aids, hand and working surface disinfection (immediately in case of contamination, otherwise following each procedure), proper disposal of contaminated materials, staff health checks, and proper staff training.

3

Taxonomy and Overview of Human Pathogenic Bacteria

Taxonomy includes the two disciplines of classification and nomenclature. The bacteria are classified in a hierarchic system based on phenotypic characteristics (morphological, physiological, and chemical characteristics). The basic unit is the species. Similar and related species are classified in a single genus and related genera are placed in a single family. Classification in yet higher taxa often takes practical considerations into account, e.g., division into "descriptive sections." A species is designated by two Latin names, the first of which denotes the genus, both together characterizing the species. Family names end in *-aceae*. Table 3.**9** provides an overview of human pathogenic bacteria.

Classification

Bacteria are grouped in the domain bacteria to separate them from the domains archaea and eucarya (see p. 5). Within their domain, bacteria are further broken down into taxonomic groups (taxa) based on relationships best elucidated by knowledge of the evolutionary facts. However, little is known about the phylogenetic relationships of bacteria, so their classification is often based on similarities among phenotypic characteristics (phenetic relationships). These characteristics are **morphological**, **physiological** (metabolic), or **chemical** (see Table 3.**8,** p. 215) in nature. The role of chemical characteristics in classification is growing in importance, for instance, murein composition or the presence of certain fatty acids in the cell wall. DNA and RNA structure is highly important in classification. DNA composition can be roughly estimated by determining the proportions of the bases: mol/l of guanine + cytosine (GC). The GC content (in mol%) of human pathogenic bacteria ranges from 25% to 70%. Measurement of how much heterologous duplex DNA is formed, or of RNA-DNA hybrids, provides information

on the similarity of different bacteria and thus about their degree of relationship. Another highly useful factor in determining phylogenetic relationship is the sequence analysis of the (16S/23S) rRNA or (16S/23S) rDNA. This genetic material contains highly conserved sequences found in all bacteria alongside sequences characteristic of the different taxa.

In formal terms, the prokaryotes are classified in phyla, classes, orders, families, genera, and species, plus subtaxa if any:

Family (familia)	*Enterobacteriaceae*
Genus	*Escherichia*
Species	*E. coli*
Var(iety) or type	Serovar O157:H7
Strain	xyz

Taxonomic classification is based on the concept of the species. Especially in an epidemiological setting, we often need to subclassify a species in **vars** or (syn.) **types**, in which cultures of a species that share certain characteristics are grouped together. Examples: biovar, phagovar, pathovar, morphovar, serovar (also biotype, phagotype, etc.). Use of the term **strain** varies somewhat: in clinical bacteriology it often designates the first culture of a species isolated from an infected patient. In an epidemiological context, isolates of the same species obtained from different patients are considered to belong to the same epidemic strain.

There is no official, internationally recognized classification of bacteria. The higher taxa therefore often reflect practical considerations.

Table 3.**9** Overview of the Medically Most Important Bacteria[1]

Family Genus, species	Characteristics	Clinical manifestations
Section 1. Gram-positive cocci		
Staphylococcaceae	Cluster-forming cocci, nonmotile; catalase-positive	
Staphylococcus aureus	Coagulase-positive, yellow-pigmented colonies	Pyogenic infections, toxicoses
S. epidermidis	Coagulase-negative, whitish colonies, normal flora	Foreign body infections
S. saprophyticus	Coagulase-negative	Urinary tract infections in young women
Streptococcaceae	Chain-forming cocci and diplococci, nonmotile, catalase-negative	
Streptococcus pyogenes	Chain-forming cocci, Lancefield group A, β-hemolysis	Tonsillitis, scarlet fever, skin infections
S. pneumoniae	Diplococci, no group antigen present, α-hemolysis	Pneumonia, otitis media, sinusitis
S. agalactiae	Chain-forming cocci, group antigen B, β-hemolysis	Meningitis/sepsis in neonates
"***Enterococcaceae***"	Chain-forming cocci and diplococci, α, β, or γ-hemolysis, group antigen D, catalase-negative	Part of the flora of intestines of humans and animals
Enterococcus faecalis *Enterococcus faecium*	Aesculin-positive, growth in 6.5% NaCl, pH 9.6	Opportunistic infections
Section 2. Endospore-forming Gram-positive rods		
Bacillaceae	Aerobic soil bacteria	
Bacillus anthracis	Nonmotile, ubiquitous	Anthrax
Clostridiaceae	Anaerobic soil bacteria	
Clostridium tetani	Motile, anaerobic, tetanus toxin (tetanospasmin)	Tetanus

[1] (Nomenclature according to Bergey's *Manual of Systematic Bacteriology*, 2001, Vol. 1, pp. 155–166. Names in quotation marks not yet validated).

Table 3.9 *Continued: Overview of the Medically Most Important Bacteria*

Family Genus, species	Characteristics	Clinical manifestations
Continued: Section 2.		
Clostridium botulinum	Motile, neurotoxins A, B, and G	Botulism, usually ingestion of toxin with food
Clostridium perfringens and further clostridiae	Nonmotile, exotoxins, and exoenzymes	1. Anaerobic cellulitis 2. Gas gangrene (myonecrosis)
Clostridium difficile	Motile, enterotoxin (toxin A), cytotoxin (toxin B)	Pseudomembranous colitis (often antibotic associated)
Section 3. Regular, nonsporing, Gram-positive rods		
Listeria monocytogenes	Slender rods, weak β-hemolysis on blood agar, motile at 20 °C, ubiquitous (soil)	Meningitis, sepsis (neonates, immunocompromised persons), epidemic gastroenteritis
Erysipelothrix rhusiopathiae	Transmitted from diseased pigs	Erysipeloid (today rare)
Gardnerella vaginalis	Flora of the normal genital mucosa	Contributes to vaginosis
Section 4. Irregular, nonsporing, Gram-positive rods		
Corynebacteriaceae	Mostly normal bacterial flora of the skin and mucosa, aerobic	Only few species cause disease
Corynebacterium diphtheriae	Club shape, pleomorphic, diphtheria exotoxin (A + B)	Diphtheria (throat, nose, wounds)
Actinomycetaceae	Normal bacterial flora of the mucosa, anaerobic or microaerophilic	
Actinomyces israelii and further *Actinomyces* spp.	Filaments (also branched)	Actinomycosis (cervicofacial, thoracic, abdominal, pelvic)
Nocardiaceae	Nonmotile, obligately aerobic, filaments, partially acid-fast	Habitat: soil and aquatic biotopes

Table 3.**9** *Continued: Overview of the Medically Most Important Bacteria*

Family Genus, species	Characteristics	Clinical manifestations
Continued: Section 4.		
Nocardia asteroides *Nocardia brasiliensis* and further species	Infections in patients with impaired cell-mediated immunity	Pulmonary, systemic, and dermal nocardioses
Section 5. Mycobacteria (acid-fast rods)		
Mycobacteriaceae	Slender rods, Ziehl-Neelsen staining (Gram-positive cell wall), aerobic, nonmotile	
Mycobacterium tuberculosis	Slow proliferation (culturing 3–6–8 weeks)	Tuberculosis (pulmonary and extrapulmonary)
Mycobacterium leprae	In-vitro culture not possible	Leprosy (lepromatous, tuberculoid)
Nontuberculous mycobacteria (NTM) (e.g., *Mycobacterium avium/intracellulare* complex, and numerous other species)	Ubiquitous. Low level of pathogenicity, opportunists	Pulmonary disease, lymphadenitis, infections of skin, soft tissue, bones, joints, tendons. Disseminated disease in immunosuppressed patients (AIDS)
Section 6. Gram-negative aerobic cocci and coccobacilli		
Neisseriaceae	Coffee bean-shaped diplococci, nonmotile, oxidase (+), catalase (+)	
Neisseria gonorrheae	Cocci often in phagocytes, acid from fermentation of glucose	Gonorrhea
Neisseria meningitidis	Acid from fermentation of glucose and maltose	Meningitis/sepsis
Eikenella corrodens	HAC**E**K-group. Low pathogenicity	Nosocomial infections
Kingella kingae	HACE**K**-group. Low pathogenicity	Nosocomial infections

Table 3.**9** *Continued: Overview of the Medically Most Important Bacteria*

Family Genus, species	Characteristics	Clinical manifestations
Continued: Section 6.		
Moraxellaceae	Cocci and short rods	
Moraxella catarrhalis	Normal respiratory tract flora	Sinusitis, otitis media in children
Acinetobacter baumannii *Acinetobacter calcoaceticus*	Ubiquitous, coccobacillary rods	Nosocomial infections, often multiple resistance against anti-infective agents
Section 7. Gram-negative facultatively anaerobic rods		
Enterobacteriaceae	Inhabitat intestine of man and animals. Genera (41) and species (hundreds) identified biochemically	
Escherichia coli	Lactose-positive, most frequent human pathogen, various pathovars.	Nosocomial infections, Gut disease caused by pathovars EPEC, ETEC, EIEC, EHEC, and EAggEC
Salmonella enterica	Lactose-negative, motile, over 2000 serovars	Typhoid/paratypoid fever, gastroenteritis
Shigella dysenteriae, S. flexneri, S. boydii, S. sonnei	Lactose-negative (in most cases), nonmotile, O-serovars	Bacterial dysentery
Klebsiella, Enterobacter, Citrobacter, Proteus, Serratia, Morganella, Providencia, and other genera	Opportunists, frequently resistant to antibiotics	Nosocomial infections
Yersinia pestis	Bipolar staining, motile, no acid from lactose. Rodent pathogen	Bubonic plague, pulmonary plague
Yersinia enterocolitica	Reservoir: wild animals, domestic animals, pets	Enteritis, lymphadenitis
Calymmatobacterium granulomatis	Encapsulated, nonmotile	Granuloma inguinale (venereal disease)

Table 3.**9** *Continued: Overview of the Medically Most Important Bacteria*

Family Genus, species	Characteristics	Clinical manifestations
Continued: Section 7.		
Vibrionaceae	Comma-shaped, polar flagella, oxidase-positive	
Vibrio cholerae	Alkaline tolerance, exotoxin, no invasion of the small intestine's mucosa	Cholera, massive watery diarrhea
Aeromonadaceae		
Aeromonas spp.	Aquatic biotopes, fish infections	Occasionally the cause of enteritis in man
Pasteurellaceae	Small straight rods, nonmotile	
Pasteurella multocida	Pathogen of various animals (sepsis)	Infections via dermal injuries (rare)
Haemophilus influenzae	X and V factors for culturing, capsule serovar "b" (Hib)	Meningitis, respiratory tract infections
Cardiobacteriaceae		
Cardiobacterium hominis	HA**C**EK group. Normal mucosal flora of humans, nonmotile	Endocarditis (rare). Opportunistic infections
Section 8. Gram-negative aerobic rods		
Pseudomonadaceae	Straight or curved rods, motile, oxidase-positive. Ubiquitous bacteria	Nosocomial infections
Pseudomonas aeruginosa and many further species	Fluorescent pigments produced. Other properties as above	Nosocomial infections, frequent multiple antibiotic resistance
"Burkholderiaceae"		
Burkholderia cepacia	Ubiquitous	Nosocomial infections. Often resistance to multiple antibiotics
B. mallei	Malleus of horses	Skin abscesses. Very rare
B. pseudomallei	Habitat: soil	Melioidosis (Asia)

Table 3.**9** *Continued: Overview of the Medically Most Important Bacteria*

Family Genus, species	Characteristics	Clinical manifestations
Continued: Section 8.		
"Xanthomonadaceae" *Stenotrophomonas maltophilia*	Low pathogenicity	Nosocomial infections. Often resistance to multiple antibiotics
Legionellaceae	Motile, difficult to stain, requires special culturing mediums	
Legionella pneumophila	Most frequent species, aquatic biotopes	Legionnaire's pneumonia, Pontiac fever
Brucellaceae	Short rods, nonmotile, facultative intracellular parasite, fastidious growth	Zoonoses
Brucella abortus *Brucella melitensis* *Brucella suis* *Brucella canis*	Transmission via direct contact or foods (milk and milk products)	Brucellosis (Bang disease, Malta fever)
Alcaligenaceae *Bordetella pertussis*	Short rods, nonmotile, only in humans	Pertussis (whooping cough)
"Francisellaceae" *Francisella tularensis*	Minute pleomorphic rods. Requires enriched media for culturing	Tularemia, zoonosis (rodents)
Section 9. Gram-negative rods, straight, curved, and helical, strictly anaerobic		
Bacteroidaceae *"Fusobacteriaceae"* *"Porphyromonadaceae"* *"Prevotellaceae"*	Pleomorphic rods, major component of normal mucosal flora	Subacute necrotic infections, mostly together with other bacteria
Bacteroides spp. *Porphyromonas* spp. *Prevotella* spp. *Fusobacterium spp.*		Necrotic abscesses in CNS, head region, lungs, abdomen, female genital tract

Table 3.**9** *Continued: Overview of the Medically Most Important Bacteria*

Family Genus, species	Characteristics	Clinical manifestations
Continued: Section 9.		
Streptobacillus moniliformis (belongs new to *Fusobacteriaceae)*	Normal flora in rats, mice, and cats	Rat-bite fever (also caused by *Spirillum minus* (= Sodoku)
Section 10. Aerobic/microaerophilic, motile, helical/vibrioid Gram-negative rod bacteria		
Campylobacteriaceae	Thin, helical, and vibrioid, culturable	
Campylobacter jejuni	Animal pathogen	Enteritis
Campylobacter fetus		Opportunistic infections: sepsis, endocarditis
"Helicobacteriaceae" *Helicobacter pylori*	Helical, culturing difficult, produces large amounts of urease	Type B gastritis, peptic ulcers of stomach and duodenum
Section 11. The Spirochetes. Gram-negative, helical bacteria		
Spirochaetaceae	Helical, motile, thin	
Treponema pallidum	Only in humans, not culturable	Syphilis, three stages
Borrelia burgdorferi *B. afzelii* *B. garinii*	Tickborne, culturable	Lyme disease, three stages
Borrelia duttonii *Borrelia hermsii* and further species	Tickborne, antigen variability	Endemic relapsing fever
Borrelia recurrentis	Transmitted by body lice	Epidemic relapsing fever
Leptospiraceae	Helical, motile, culturable	
Leptospira interrogans	Serogroups and serovars (e.g., icterohemorrhagiae, pomona, grippotyphosa, etc.)	Leptospirosis, morbus Weil

Table 3.**9** *Continued: Overview of the Medically Most Important Bacteria*

Family Genus, species	Characteristics	Clinical manifestations
Section 12. Rickettsiae, Coxiellae, Ehrlichiae, Bartonellae, and Chlamydiae		
Rickettsiaceae	Small short rods, usually intracellular bacteria transmitted by arthropods	Rickettsioses
Rickettsia prowazekii	Transmitted by body lice	Typhus
Rickettsia rickettsii	Transmitted by ticks	Rocky Mountains Spotted Fever (RMSF)
"Coxelliaceae" *Coxiella burnetii*	Reservoir: sheep, cattle, rodents; infection by inhalation	Q fever (pneumonia)
Ehrlichiaceae	Coccobacillary. Culture possible	Zoonoses
Ehrlichia chaffeensis	Transmission by ticks	Human monocytrophic ehrlichiosis (HME)
Ehrlichia ewingii and Anaplasma (formerly Ehrlichia) phagocytophilum	Transmission by ticks	Human granulocytotrophic ehrlichiosis (HEG)
Bartonellaceae	Short pleomorphic rods	
Bartonella bacilliformis	Tropism for erythrocytes/endothelia. Transmitted by sand flea	Oroya fever and verruga peruana
Bartonella henselae and *Bartonella claridgeia*	Animal reservoir: cats	Sepsis, bacillary angiomatosis in immunosuppressed patients (AIDS). Cat scratch disease in immunocompetent persons
Bartonella quintana	Transmission by body lice	Five-day fever
Chlamydiaceae	Obligate intracellular pathogen, reproductive cycle	
Chlamydia trachomatis	Biovar trachoma	Trachoma, inclusion conjunctivitis, urethritis (nonspecific)
	Biovar lymphogranuloma venerum	Lymphogranuloma venereum

Table 3.**9** *Continued: Overview of the Medically Most Important Bacteria*

Family Genus, species	Characteristics	Clinical manifestations
Continued: Section 12.		
Chlamydia psittaci	Reservoir: infected birds. Infection by inhalation of pathogen-containing dust	Ornithosis (pneumonia)
Chlamydia pneumoniae	Only in humans, aerogenic transmission	Infections of the respiratory tract, often subacute. Role in atherosclerosis of coronary arteries still unclear
Section 13. Mycoplasmas (bacteria without cell walls)		
Mycoplasmataceae	Pleomorphic; no murein, therefore resistant to antibiotics that attack the cell wall	
Mycoplasma pneumoniae	Reservoir human, aerogenic infection	Pneumonia (frequently atypical)
Ureaplasma urealyticum	Component of the normal flora of the urogenital tract	Urethritis (nonspecific)

Nomenclature

The rules of bacterial nomenclature are set out in the *International Code for the Nomenclature of Bacteria*. A species is designated with two latinized names, the first of which characterizes the genus and the second the species. Family names always end in *-aceae*. Taxonomic names approved by the "International Committee of Systematic Bacteriology" are considered official and binding. In medical practice, short handles have become popular in many cases, for instance gonococci instead of *Neisseria gonorrheae* or pneumococci (or even "strep pneumos") instead of *Streptococcus pneumoniae*.