

Viruses

General. A virus is an infectious particle without indigenous metabolism. Its genetic program is written in either DNA or RNA, whose replication depends on the assistance of a living host cell. A virus propagates by causing its host to form a protein coat (capsid), which assembles with the viral nucleic acid (virus particle, nucleocapsid). Viruses can infect most living organisms; they are mostly host-specific or even tissue- or cell-specific. Viruses are classified by their host range, their morphology, their nucleic acid (DNA/RNA), and their capsids. In medicine and veterinary medicine, the early diagnosis, prophylaxis and therapy of viral human and veterinary diseases plays a crucial role. AIDS (HI virus), viral hemorrhagic fever (Ebola virus), avian flu (H5N1-, H7N9-virus) ($\rightarrow 250$) or hepatitis (several virus families) are important examples of viruses involved in human diseases, as are Rinderpest (Morbillivirus) or infectious salmon anemia (ISA virus) in epizootic veterinary diseases. In biotechnology, viruses are used for the development of coat-specific or component vaccines and for obtaining genetic vector and promoter elements which are, e.g., used in animal cell culture and studied for use in gene therapy.

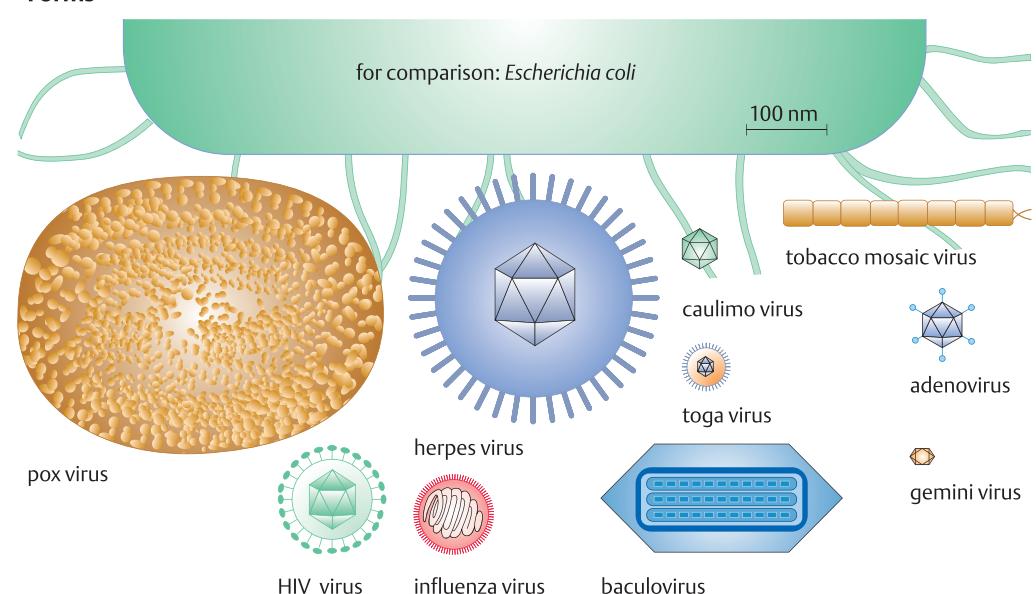
Viruses for animal experiments. The first cloning experiments with animal cells were done in 1979, using a vector derived from simian virus 40 (SV40) ($\rightarrow 98$). This virus can infect various mammals, propagating in lytic or lysogenic cycles (lysis vs. retarded lysis of host cells). Its genome of ca. 5.2 kb contains early genes for DNA replication and late genes for capsid synthesis. Expression vectors based on SV40 contain its origin of replication (ori), usually also a promoter, and a transcription termination sequence (polyA) derived from the viral DNA. For the transfection of mouse cells, DNA constructs based on bovine papilloma virus (BPV) are preferred. In infected cells, they change into multicopy plasmids which, during cell division, are passed on to the daughter cells. Attenuated viruses derived from retro, adeno, and herpes viruses are being investigated as gene shuttles for gene therapy ($\rightarrow 304$). Retroviruses, e.g., the HI virus, contain an RNA genome. They infect only dividing cells and code for a reverse transcriptase which, in the host cell, transcribes the

RNA into cDNA. HIV-cDNA is then integrated into the host genome where it directs, via strong promoters, the formation of viral nucleic acid and capsid proteins. Some hundred experiments with retroviral vectors having replication defects have already been carried out for gene therapy. A disadvantage of using retroviral vectors lies in their small capacity to package foreign DNA (inserts), whereas vectors derived from adenoviruses can accommodate up to 28 kb of inserted DNA. In contrast to retroviruses, adenoviruses can infect non-dividing cells, but their DNA does not integrate into the host chromosomal DNA. For gene therapy targeted to neuronal cells, e.g., in experiments related to Alzheimer's or Parkinson's disease, *Herpes simplex*-derived vectors are often used. Their large genome of 152 kb allows them to accommodate inserts > 20 kb of foreign DNA. A similar insert size is reached with Vaccinia viruses, which may infect a wide range of cell types.

Viruses for plant experiments. Most plant viruses have an RNA genome ($\rightarrow 280$). Only two groups of DNA viruses are known that infect higher plants, caulimo virus and gemini virus. Caulimo viruses have a quite narrow host range: they infect only crucifers such as beets and some cabbage varieties. Their small genome reduces their potential for accommodating foreign inserts. Gemini viruses infect important agricultural plants such as maize and wheat and thus bear significant risks for application. Moreover, their genomes undergo various rearrangements and deletions during the infection cycle, rendering the correct expression of foreign DNA inserts difficult.

Baculoviruses infect insects but not mammals. After infection, host cells form a crystalline protein (polyhedrin), which may constitute $> 50\%$ of the insect cell. The polyhedrin promoter is therefore useful for the heterologous expression of proteins, using cell cultures of *Spodoptera* (a butterfly). An advantage of this system is that posttranslational glycosylations in this system resemble those of mammalian cells ($\rightarrow 262$). Scale-up of this system is, however, limited, rendering it most useful for laboratory experiments. In Japan, silk worms (*Bombyx mori*) are considered an interesting system for expressing foreign proteins. The nuclear polyhedral virus BmNPV is being used for their transfection.

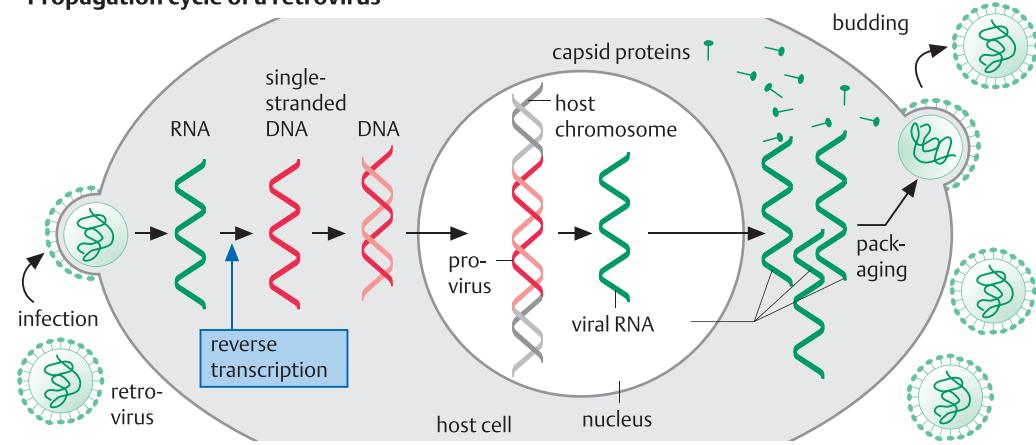
Forms



virus	host	disease	capsid	nucleic acid
smallpox	man, cattle	smallpox	complex coat	linear DNA, d
hepatitis B	man	hepatitis B	polyhedral capsid	circular DNA, d
toga	man	measles	polyhedral capsid	(+)-RNA, s
herpes	man, birds	belt rose, herpes	polyhedral capsid	linear DNA, d
HIV	man, primates	AIDS	round capsid	2 × (+)-RNA, s
influenza	man, mammals	influenza	helical coat	(-)RNA, segmented
adenovirus	man	common cold	polyhedral capsid	linear DNA, d
papilloma	cattle	warts	polyhedral capsid	circular DNA, d
tobacco mosaic	tobacco plant		polyhedral capsid	RNA, s
caulimo	cabbage		polyhedral capsid	circular DNA, s
gemini	dicots		double polyhedron	circular DNA, s
baculo	insects		polyhedral capsid	circular DNA, d

s = single strand, d = double strand, + = sense direction, - = antisense direction

Propagation cycle of a retrovirus



Bacteriophages

General. Viruses that attack bacteria are termed bacteriophages or simply phages. Their taxonomy is determined by the International Committee on Taxonomy of Viruses, ICTV. Phages occur everywhere in nature, and are abundant in metagenomic analyses of water samples (→74). As there are historic reports of healing by “holy waters,” they have been widely studied for the treatment of bacterial infections but results are equivocal. Fermentation processes, e.g., starter culture production (→114), are always endangered by phage infections. As a preventive measure, attempts are usually made to select phage-resistant strains. Phages are useful in genetic engineering, e.g., for the development of cloning vectors or promoters, for DNA sequencing, and for the preparation of gene and protein libraries (→62, 64, 68). Since most cloning experiments use *E. coli*, phages specific for this bacterium (λ -, M13-, Q β -, T-phages) play a key role.

λ Phage. When infecting *E. coli*, λ phage can follow two routes: either its linear double-stranded DNA (ca. 48.5 kbp, ca. 1% of the *E. coli* genome) is propagated independent of the *E. coli* genome, resulting in lysis (lytic cycle), or it is integrated into the *E. coli* genome, resulting in lysogenic cells containing latent prophages, which replicate with the bacterium over several generations. Upon stress, such as a rise in temperature or UV irradiation, the prophage is excised from the *E. coli* genome and lyses the host cell. λ is able to form cohesive or sticky ends of 12 unpaired nucleotides each (cos sites), which are necessary for circular λ DNA formation and for its integration into the *E. coli* genome. The sticky ends also form the recognition signal for the formation of the viral gene product A, an exonuclease. After replication of the λ DNA into a concatemer of linear λ genomes, endonuclease A cuts at this position, initiating the packaging of progeny into its capsids. Cosmids, an important tool for the construction of large gene libraries, are derived from the λ phage, as is a family of λ plasmids such as λ EMBL4, which can be induced by a rise in temperature.

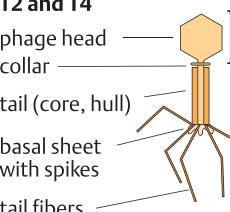
The M13 phage infects *E. coli* according to a different mechanism. It contains single-strand-

ed DNA of ca. 6.4 kb, which after infection directs the synthesis of its complementary strand. The resulting double-stranded phage DNA is not integrated into the *E. coli* genome but is continuously replicated in the cytoplasm, giving rise to up to 1,000 phage particles/cell. During host cell division, the phage infection is passed on to the daughter cells (ca. 100/cell). Genes that have been cloned into a vector derived from M13 can be obtained as single-stranded DNA – a technique used for classical DNA sequencing (→56). Prior to the invention of PCR, M13 vectors were used for site-directed mutagenesis of proteins.

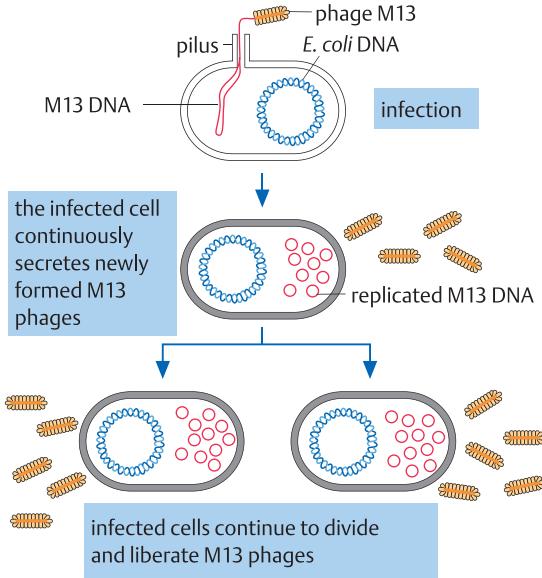
T Phages occur in 7 different types. For genetic engineering, two enzymes coded by T phage genomes are useful: the DNA ligase of T4, which links DNA fragments regardless of the quality of their ends (sticky or blunt), and the DNA polymerase of T7, which polymerizes DNA on a single strand DNA matrix; it is used in gene sequencing (Sanger–Coulson method). The promoter of the T7-RNA polymerase is used in several *E. coli* expression vectors. T7-RNA polymerase transcribes DNA into RNA, which in turn serves as mRNA in cell-free protein synthesis, based on mRNA, tRNAs, ribosomes, amino acids and ATP.

Phages of other bacteria. Among the > 1,000 classified phages (some 2800 in total), > 300 are specific for enterobacteria, > 230 for bacilliocci, and > 150 each for Bacilli and Actinomycetes. Another group (at present 13 phages), described only recently, is the Ligamenvirales which attack archaeabacteria. Their structure and function are closely related to those of other viruses, including those specific for *E. coli*. Some of them can be either virulent or lysogenic, similar to the λ phage. Lactobacilli-specific phages are a major problem in the manufacture of milk products. Resistant bacteria prevent adsorption or replication of these phages. Among the 5 groups of *Bacillus* phages, ϕ 105 and SPO2 are often used for transformation, and PBS1 has been used in construction of the *B. subtilis* genome sequence map. Phage D3112 is the preferred vector for the transformation of Pseudomonads, and SH3, SH5, SH10, or ϕ C31 are preferred for the genetic engineering of Streptomyces.

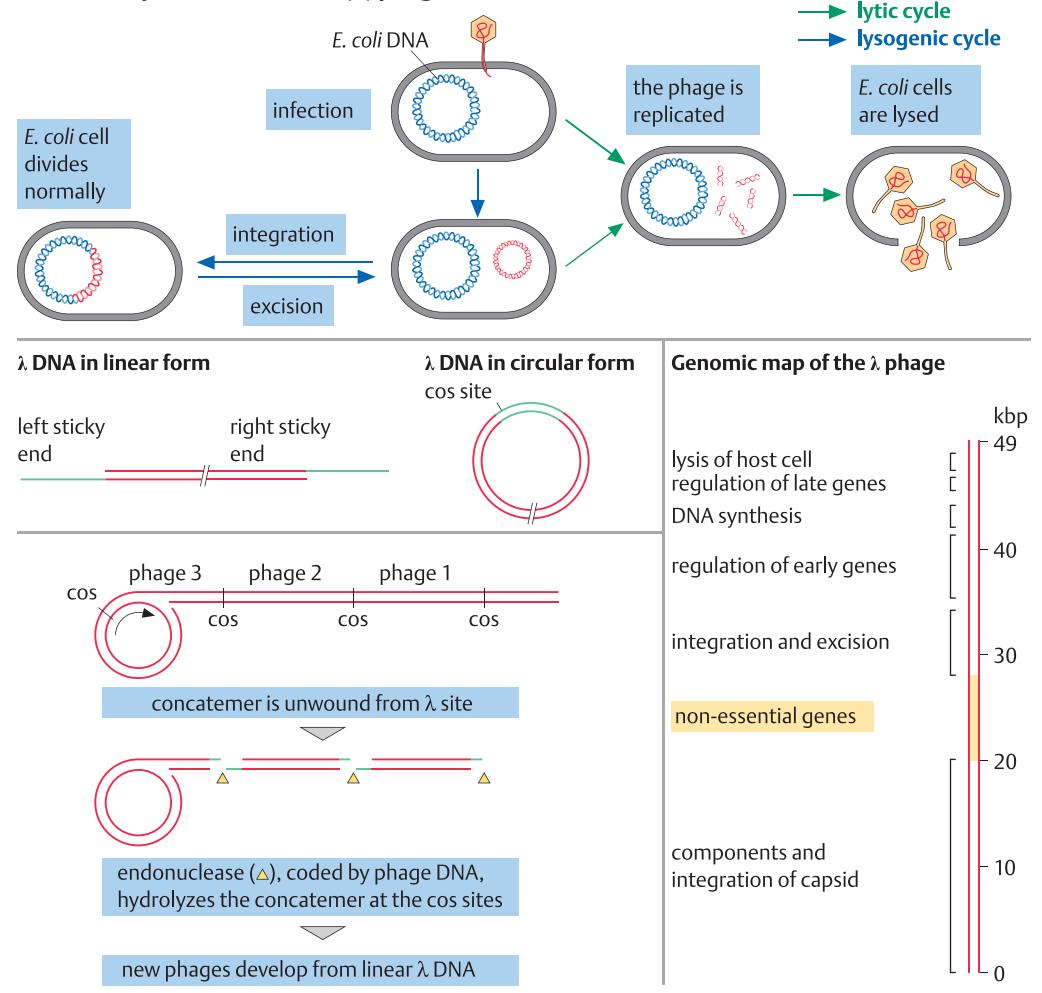
E. coli phages (select)

name	form	genetic material
T2 and T4	 phage head collar tail (core, hull) basal sheet with spikes tail fibers	DNA (double-stranded)
T7		DNA (double-stranded)
lambda (λ)		DNA (double-stranded)
M13	 6 nm 900 nm	DNA (single-stranded)

Infection cycle of M13



Infection cycle of the lambda (λ) phage



Microorganisms

General. Microorganisms play a key role in the chemical cycles on earth. They are involved in the biodegradation of many compounds; these processes occur not only in the environment, but also in symbiosis with other organisms (e. g., lichens, intestinal and rumen bacteria). Some microorganisms are parasites or pathogens, impairing the health or life of other organisms. In biotechnology, nonpathogenic microorganisms are used to produce various products such as citric and glutamic acid, antibiotics, xanthan, and enzymes; for the aerobic and anaerobic treatment of wastewater, sludges, soils, and air; and as host organisms for the manufacture of recombinant proteins. Due to their unicellular structure, well established methods for creating and selecting mutants, and their short generation time, they serve as model organisms for understanding the biochemical, genetic, and physiological mechanisms of life, and as a preferred host for the manufacture of recombinant proteins. Based on some fundamental differences, prokaryotic and eukaryotic microorganisms can be distinguished; the former are further subdivided into eubacteria and archaeabacteria (> 10,000 different fully characterized strains).

Eubacteria are unicellular organisms that propagate by cell division. Their cell diameter is usually on the order of 1 μm . They have no cell nucleus, and their chromosomal DNA is formed into a tangle, the nucleoid. Frequently, part of their genetic makeup occurs on nonchromosomal genetic elements, the plasmids (\rightarrow 44). Plasmids are often horizontally transferred to other bacteria – a useful mechanism, from the human perspective, for evolving biodegradation pathways for xenobiotic compounds in the environment and sewage plants, but a very dangerous capacity with respect to the evolution of antibiotic resistances. The cell wall, made of peptidoglycan, is more complex in Gram-negative microorganisms and often covered with a slimy layer from which flagella may protrude, which ensure mobility. In the cytoplasm, storage chemicals such as polyhydroxybutyric acid polyphosphate, cyanophycin, or others may be deposited. Eubacteria have a wide potential for variations in metabolism and thus can grow in a much wider range of habitats than higher organisms. Such highly specialized species often surprise us by their unique proteins and

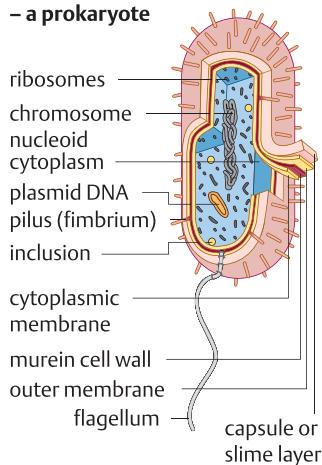
cofactors. Thus, the purple membrane of the halobacteria is a unique functional unit of this genus, exhibiting some analogies to photosynthesis and the chemistry of vision in higher organisms.

Archaeabacteria (archaea) are believed to resemble the oldest forms of life on earth. Their footprints have been detected in geological formations many hundreds of millions of years old. They often live anaerobically and are usually specialized for growth in unique biotopes. As just one example, the methanobacteria form the most important group of sludge consortia, reducing acetic acid to methane (\rightarrow 288). They differ from the eubacteria in structural and genetic properties, e. g., in the construction of their cell membrane from ether lipids instead of phospholipids. The function of their enzymes is adapted to their often extreme habitats and have been used in biotechnology. For example, a DNA polymerase from a deep-sea bacterium, *Pyrococcus furiosus*, is often used for PCR reactions with particular high fidelity (\rightarrow 50, 196).

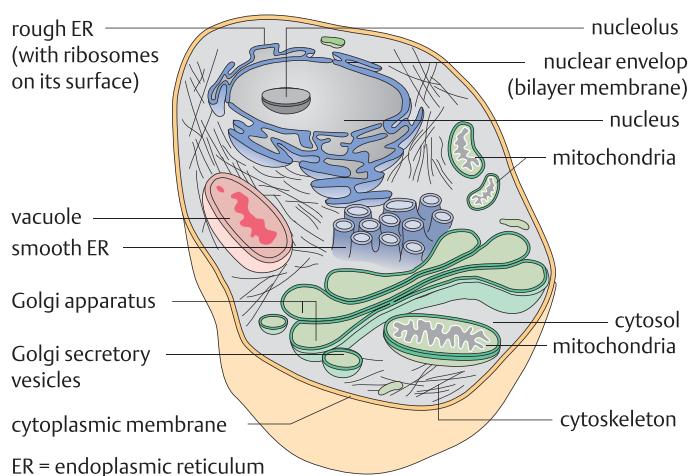
Yeasts and fungi are eukaryotic organisms and so far constitute the largest group of cultivatable microorganisms: about 70,000 different strains have been taxonomically classified. In contrast to prokaryotes, they contain a cell nucleus and other subcellular functional units, and their cell wall is made of chitin, sometimes also from cellulose. Most yeasts and fungi live aerobically. Their wide differences in reproduction and life cycles provide the most useful basis for their taxonomic classification. The vegetative body of fungi is composed of a hairy network, the mycelium, which can propagate sexually or asexually. Asexual reproduction usually proceeds by spore formation, or occasionally by budding. Sexual reproduction of the lower fungi (Phycomycetes) proceeds via gametes, of the higher fungi via fruiting bodies (asci) which have the form of a sac (Ascomycetes) or a club (Basidiomycetes). Yeasts (e. g., *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*) and fungi (e. g., *Aspergillus oryzae*, *Trichoderma viride*) are frequently used hosts for the manufacture of recombinant enzymes and other proteins. Unlike prokaryotic hosts, they perform post-translational modifications such as glycosylation (\rightarrow 262), an often important feature for the production of pharmaceutical proteins (glycobiology).

Microorganisms

Escherichia coli – a prokaryote

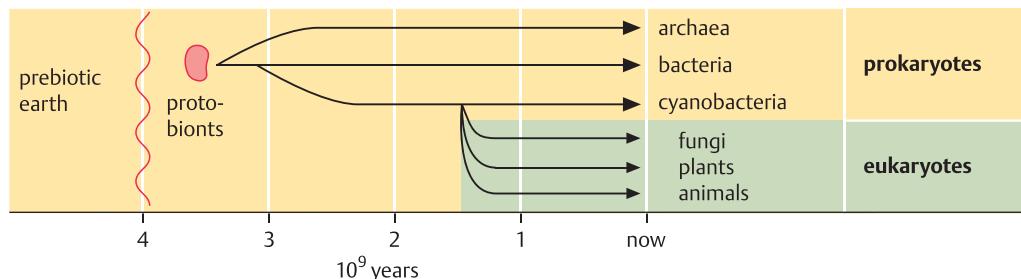


Saccharomyces cerevisiae – a eukaryote



	<i>E. coli</i>	<i>S. cerevisiae</i>	for comparison: plant and animal cells
cell nucleus, organelles	no	yes	yes
diameter [μm]	~ 1	~ 10	~ 100
volume [μm^3]	~ 1	~ 1000	$>10\,000$
respiration [$\mu\text{L O}_2/\text{mg TS} \cdot \text{h}$]	1000	100	10
generation time [h]	0.3	1.5	>20
genes	$\sim 4\,300$	$\sim 6\,000$	$>30\,000$

Position of the microorganisms in evolution



Archaea, eubacteria, and lower eukaryotes

	archaea	eubacteria	fungi, yeasts
cell type	prokaryote	prokaryote	eukaryote
cell wall	heteropolysaccharide or glycoprotein	peptidoglycan	glucan, chitin
membrane lipids	ether lipids from isoprenoid building blocks	phospholipids	phospholipids
initiator tRNA	methionine	formyl methionine	methionine
genetic material	small circular chromosome, plasmids, histone-type proteins	small circular chromosome, plasmids	complex nucleus with > 1 chromosome and linear DNA, histones
RNA polymerase	complex	simple	complex
size of ribosomes	70S	70S	80S

Bacteria

General. Bacteria can be classified by a variety of morphological, biochemical, and genetic methods, as well as by their nutrient requirements. The *International Code of Nomenclature of Bacteria* (ICNB) governs the scientific naming of bacteria and presently includes about 2,200 genera and 11500 species. The analysis of taxonomically relevant DNA isolated from soil seems to indicate, however, that the number of bacterial species that have not yet been cultured is much larger (→74).

Eubacteria. The oldest method of classifying eubacteria is based on their morphology. Under a simple light microscope, rods, cocci, and spirilli can be seen, some of them forming multicellular aggregates (filaments, colonies) and exhibiting structural details such as spores or flagella. Staining provided further differentiation. Thus, staining according to H. C. Gram's method allows for a classification according to cell wall structure: Gram-positive bacteria have only one cell membrane, covered by a thick murein cell wall, whereas Gram-negative bacteria have two cell membranes, enclosing a periplasmic space. The outer membrane is covered by a thin murein cell wall from which lipopolysaccharides may protrude. Physiological and biochemical criteria have led to additional methods of differentiation. Some important features are:

Response to oxygen: microorganisms can be subdivided according to their ability to grow under aerobic, anaerobic, or both conditions,
Form of energy generation: energy can be generated by photosynthesis (phototrophs), respiration, or fermentation (chemotrophs),

Preferred electron donors: organotrophic microorganisms use organic compounds, and lithotrophic microorganisms use inorganic compounds such as H_2 , NH_3 , H_2S , CO , or Fe^{2+} .

Carbon source: autotrophic microorganisms can fix CO_2 ; heterotrophic microorganisms obtain carbon from organic compounds,

Relation to other organisms: saprophytic microorganisms are autonomous; parasitic microorganisms depend on a host organism.

Phage typing: the susceptibility to phages can also be used for taxonomic identification,

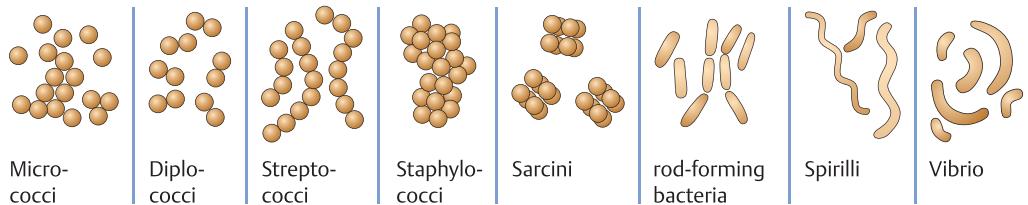
Adaptation to environment: mesophilic microorganisms grow under ordinary conditions, whereas extremophiles are adapted to extreme conditions of temperature, pressure, pH, or

electrolyte concentration. Cell inclusions, pigments, chemical components of the cell wall and cell membrane (fatty acid composition), immunological differentiation of the cell surface (serology), and susceptibility to antibiotics provide further possibilities for phenotype differentiation. Recently, genotyping of bacteria has become more and more important. For example, the GC content of bacterial DNA enables a rough classification. Complete sequencing of microbial genomes enables the most precise differentiation. A particularly useful method for taxonomy, discovered in 1972, is sequencing the DNA coding for the 16S, 18S and 23S rRNA (S: Svedberg units characterizing sedimentation behavior). This DNA contains sequences that were highly conserved throughout evolution, and analyses of the sequences suggest three families of living organisms: archaeabacteria, eubacteria (the prokaryotes), and the eukaryotes. If DNA is isolated from environmental samples, and sequences coding for 16S, 18S or 23S rRNA are compared to those of microorganisms deposited in culture collections, there is less than 5 % identity, suggesting that > 95 % of all microorganisms contained in these samples have not yet been cultivated (s. metagenome) (→74).

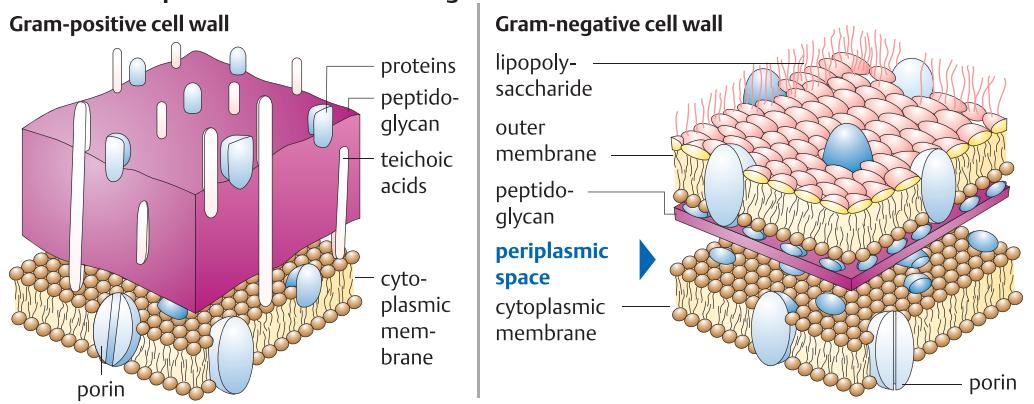
Characterization and taxonomy. Rapid taxonomic identification of bacteria is important in hospitals, veterinary medicine, food production, environmental hygiene, and also in microbial and genetics laboratories. Most of the above methods are used, e. g., microscopy, staining procedures, determining the "analytical profile index API" (based on growth on various substrates), fatty acid composition of the membrane, or DNA analysis of taxon-specific sequences coding for the 16S, 18S or 23S rRNA. Precise classification of microorganisms is often far from trivial and requires the consideration of a wide range of experimental data; it is usually done by laboratories that archive culture collections.

Genome sequencing. As of 2013, genome sequences for some 2,100 bacteria and over 140 archaea are completed. This includes many genomes of human pathogens such as *Mycobacterium tuberculosis*. The analysis of microbial genomes has shown that many variations of metabolic pathways exist, which can be exploited by metabolic engineering.

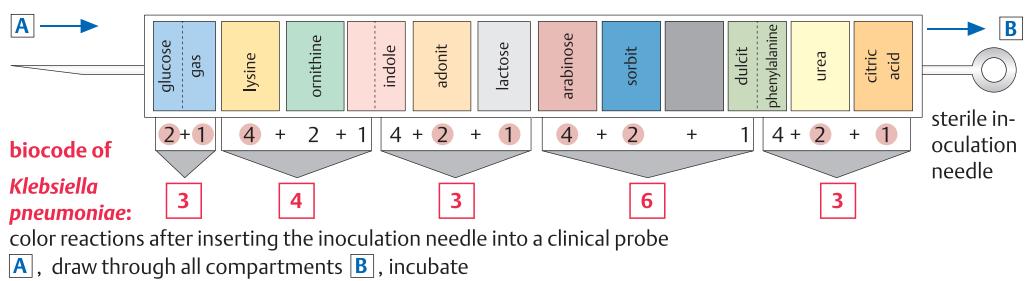
Forms of unicellular bacteria



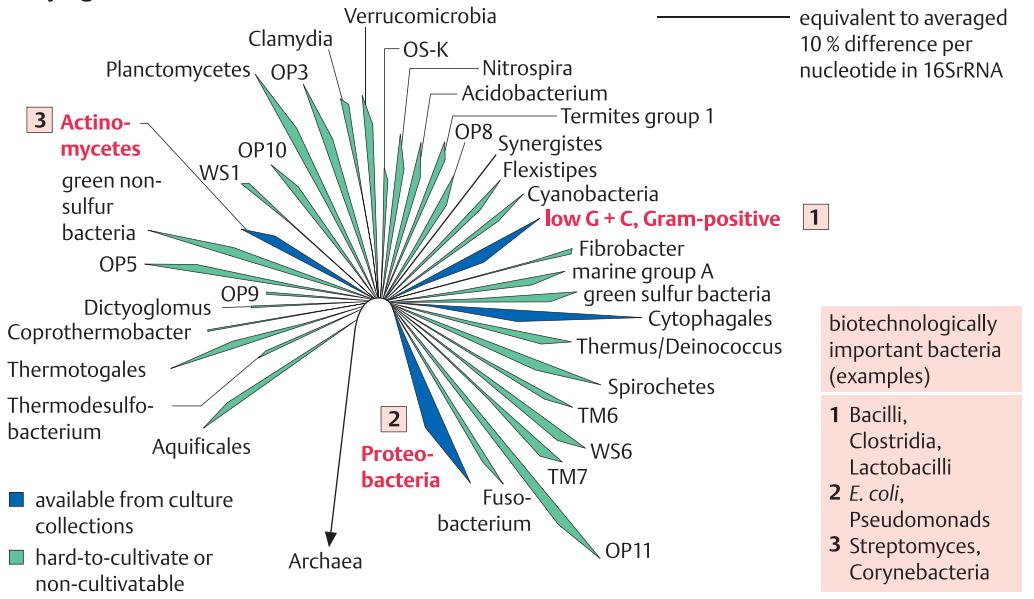
Cell wall composition and Gram-staining



Biochemical characterization



Phylogenetics and cultivation



Yeast

General. Yeasts are a subgroup of the Ascomycetes. Because they propagate by budding, they are also termed budding fungi. They grow heterotrophically, preferring acidic media (pH 3.5–5.0) and usually do not form mycelia. Their cell wall is made of chitin. *Candida albicans* is an important human pathogen and model for studying pathogenesis. Yeasts of importance for biotechnology are *Saccharomyces cerevisiae*, *Candida utilis* and other *Candida* strains, *Schizosaccharomyces pombe*, *Hansenula polymorpha*, and *Pichia pastoris*.

Saccharomyces cerevisiae (synonyms: baker's yeast, brewer's yeast, yeast) (→120) can propagate in either a haploid or diploid manner, thus providing an excellent organism for genetic investigations. Haploid laboratory strains belong to one of two mating types (*MAT α* or *MAT α*), which can only mate reciprocally. Asexual reproduction proceeds by forming conidia, followed by immigration of either a diploid or a haploid nucleus. Sexual propagation occurs by the fusion of two haploid gametes, followed by meiosis and formation of 4 haploid ascospores, whose phenotype can be separately observed, allowing for simple genetic analysis of the observed traits (tetrad analysis). Due to the simple cultivation of both haploid and diploid cells, the completed genome sequence (12 Mbp, on 16 chromosomes), the general absence of introns, and the short doubling time (90 min), *S. cerevisiae* has become a preferred model organism for the molecular genetics of a simple eukaryote. Another advantage is that yeast occurs with a natural plasmid, termed 2 μ m (60–100 copies in the cell nucleus), and that a second extrachromosomal element, the killer virion, is also available for recombination experiments. Many cloning vectors have been developed for yeast transformation, which either allow the replication of foreign genes outside the yeast chromosome (YRP = yeast replicating plasmids or YEP = yeast episomal plasmids) or integration of the foreign gene into the chromosome (YIP = yeast integrating plasmids). Artificial yeast chromosomes (YAC = yeast artificial chromosomes) allow for the cloning of large DNA fragments of 600–1,400 kbp; they have been widely used for preparing genome libraries, but have a tendency to recombine and thus have been mostly replaced by bacte-

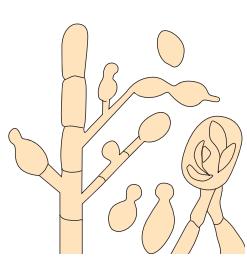
rial artificial chromosomes (BACs) (→72). The ca. 6,000 genes of yeast, located on 16 linear chromosomes, often show high homology to human genes. Thus yeasts have widely served as a simple model system for metabolic and regulation studies. In biotechnology, yeasts are used in the preparation of food products such as beer (→112), wine (→110), and bread (→120). It is also used in the manufacture of industrial ethanol (→138). Recombinant yeasts have become important host organisms for the manufacture of products such as insulin (→222), interferons (→234), and vaccines (→250) (e.g., hepatitis B surface antigen). Unlike *E. coli*, yeast allows for the posttranslational modification of gene products, in particular for glycosylation (→262).

Candida utilis differs from *Saccharomyces* by forming a mycelium, but it propagates solely asexually by budding. Some *Candida* genes show noncanonical codon usage (e.g., CUG for serine instead of leucine), which has retarded their heterologous expression. *Candida* strains have been used in biotechnology for production of extracellular enzymes and generation of digestible biomass. They can be grown on unconventional substrates such as sulfite suds or alkane fractions. Some *Candida* strains, such as *Candida albicans*, are pathogenic to humans.

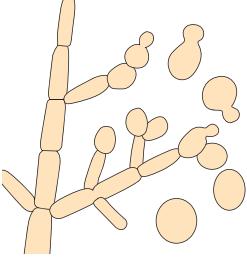
Pichia pastoris* and *Hansenula polymorpha are methylotrophic yeasts, which can grow on methanol as their sole carbon source. Isolated and studied in the context of the manufacture of single-cell protein (→122), they are used today as attractive host organisms in cloning experiments. Thus, diverse proteins such as lipases, β interferon, and antibody fragments have been functionally expressed in *P. pastoris* in yields of several grams of recombinant products/L of culture broth. The *Hansenula polymorpha* genome (9.5 Mbp, 6 chromosomes) was sequenced in 2003, the *Pichia pastoris* genome (9.4 Mbp, 4 chromosomes) was sequenced in 2009.

Schizosaccharomyces pombe was first isolated from an East African beer variety (Swahili: pombe = beer). The genome of this ascomycete was fully sequenced in 2002 (12.6 Mbp, 3 chromosomes), and is similar in size to the *S. cerevisiae* genome. Mutant strains with reduced genome size and partial deletion of protease genes have been constructed which allow for excellent expression of foreign proteins.

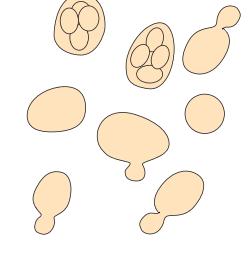
Morphology



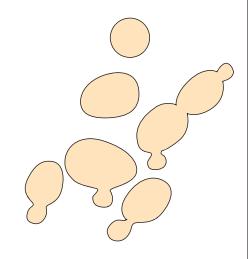
Endomycopsis
forms ascus
forms mycelium
forms buds



Candida
-
forms mycelium
forms buds



Saccharomyces
forms ascus
-
forms buds



Torulopsis
-
-
forms buds

genetic

	size of haploid genome [Mbp]	chromosomes	gene	genome-sequence
<i>Saccharomyces cerevisiae</i>	12.1	16	5905	1996
<i>Candida utilis</i>	14.6	14	8646	2012
<i>Pichia pastoris</i>	9.4	4	5040	2009
<i>Hansenula polymorpha</i>	9.5	6	5933	2003
<i>Schizosaccharomyces pombe</i>	14.1	3	4970	2002
for comparison: <i>Escherichia coli</i> K12	4.6	1	4145	1997

Technical applications of yeasts

Saccharomyces cerevisiae

- baker's yeast, brewers yeast
- host organism for the expression of peptides, proteins and enzymes
- model organism for the analysis of metabolic and gene regulation
- model organism for aging research

Candida strains

- animal feed
- manufacture of biosurfactants
- biotransformation reactions

Pichia pastoris, *Hansenula polymorpha*

- host organisms for the expression of proteins and enzymes

Schizosaccharomyces pombe

- host organism for the expression of proteins and enzymes
- model organism for the analysis of gene regulation

Yeast

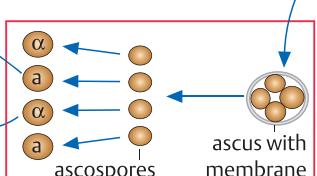
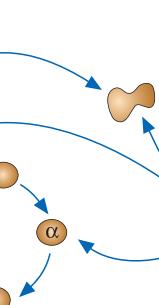
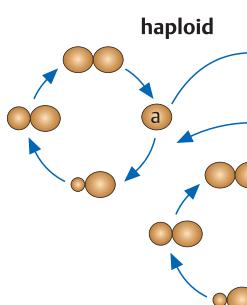


Schizosaccharomyces pombe



Saccharomyces cerevisiae
= baker's yeast

Reproduction cycle of *S. cerevisiae*



zygote

ascospores



ascus with membrane

diploid
tetrad
analysis
inheritance
follows
Mendelian
rules



Fungi

General. Fungi play a key role in the carbon catabolism of the biosphere, e.g., in the decomposition of wood and the formation of humic acids. Mycorrhizal fungi are associated with plant roots and assist in the uptake of nutrients, but other fungi, such as mildews, are dangerous plant pathogens. In biotechnology, they have an important role in the decay of food, but also in the preparation of fermented food products. Some fungi produce antibiotics or valuable enzymes. Among ca. 70,000 fungal species that have been classified, the Ascomycetes comprise ca. 20,000 species, forming the largest subgroup, which includes *Penicillium notatum* and *Aspergillus niger*. Among the lower fungi (Zygomycetes), *Rhizopus* and *Mucor* species have the greatest importance in biotechnology. Some of the ca. 12,000 stand mushrooms (Basidiomycetes) are edible (e.g., chameignons, shiitake, chanterelles, ceps), and others participate in the degradation of wood (white and red rot fungi). Approximately 300 fungal species are pathogenic to humans. All fungi live heterotrophically. Their cell wall is composed of chitin and glucans.

Reproduction forms. The reproduction of fungi follows highly diverse patterns, which are described here using the Ascomycetes as an example. The cell mass (thallus) consists of a mycelium that is made up of hyphae. During asexual reproduction, the conidiophores, which form at the top of the mycelium, divide and form spores (conidia), which grow into a new mycelium. Like most fungi, Ascomycetes can also propagate by a sexual mechanism. This results in a different phenotype (dimorphism). In this case, their hyphae form male and female sexual organs (antheridia and ascogonia). They fuse, during plasmogamy, into dikaryotic hyphae, which develop into an ascocarp ("fruiting body"). In the terminal cells of the dikaryotic hyphae, the dikaryotic nuclei are fused into a diploid zygote (karyogamy). Meiosis transforms the zygote into 8 haploid ascospores (or 4 basidiospores, in Basidiomycetes), which again grow into a mycelium.

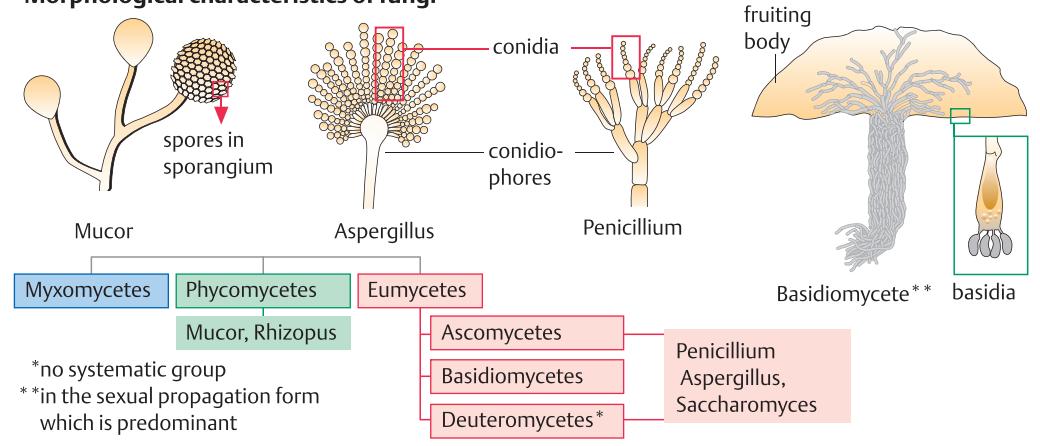
Penicillium chrysogenum grows as a mycelium which forms fruiting bodies liberating spores (conidia) for asexual reproduction. Fungi like *Penicillium*, which have lost the capacity for sexual reproduction, are termed

Fungi imperfecti. Consequently, if recombination is required during breeding in the laboratory, protoplast fusion among different types of nuclei (heterokaryosis) must be used. *P. chrysogenum* and the related fungus *Acremonium chrysogenum* are important industrial organisms, since they synthesize the lactam antibiotics (→206). Other *Penicillium* species such as *Penicillium camembertii* play an important role in the maturation of cheese (→188). The genome of *P. chrysogenum* contains ca. 32 Mbp and the sequence was published in 2008.

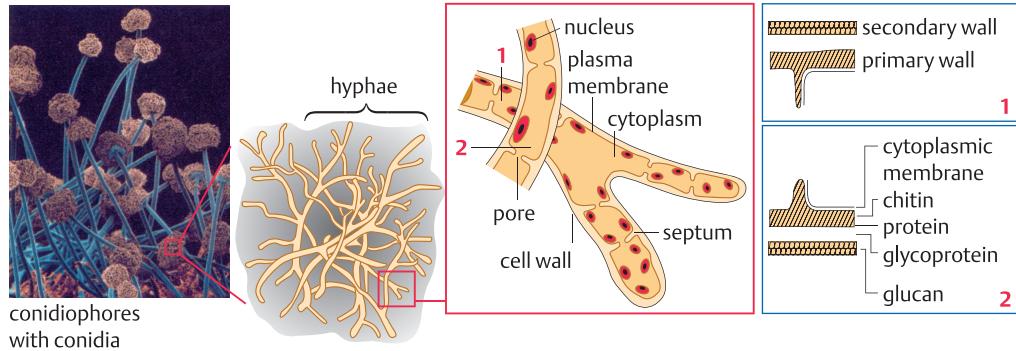
Aspergillus nidulans differs from *Penicillium* in the form of its conidia. Its genome contains 30.5 Mbp. *A. oryzae* is used for industrial production of extracellular enzymes (→172) and is a favorite host organism for producing recombinant enzymes from other eukaryotes. Various *Aspergillus* strains play a traditional role in Asian countries for the manufacture of food products such as soy sauce, miso, and sake (→86, 114), and their genetic and biochemical properties related to the production of these products have been analyzed in great detail. *Aspergilli* are also used for the production of extracellular enzymes such as proteases or amylases, and are preferred hosts for the production of recombinant fungal enzymes which they secrete. *A. niger* is the preferred production organism for citric and gluconic acid (→146, 150). Similar to *Penicillium*, strain improvement still uses protoplast fusion and selection; as the genome sequences of *A. nidulans*, *A. niger*, *A. oryzae* and eight more *Aspergillus* strains are now available (2013), targeted strain improvements based on the molecular genetic analysis of desired traits are rapidly advancing.

Rhizopus oryzae, a zygomycete, grows on rice, and *R. nigricans* is the black mold on bread. Its hyphae grow rapidly and bore their way through their substrates. Asexual reproduction proceeds by the formation of spores in differentiated mycelium (sporangia). *Rhizopus* and the closely related *Mucor* species can also grow on decaying organic materials and synthesize numerous extracellular hydrolases for this purpose. As a result, they have become important organisms for the manufacture of extracellular enzymes such as lipases and proteases. The *R. oryzae* genome is composed of 45.2 Mbp and was completely sequenced in 2009. A second *Mucor* genome sequence is available from *Mucor circinelloides*.

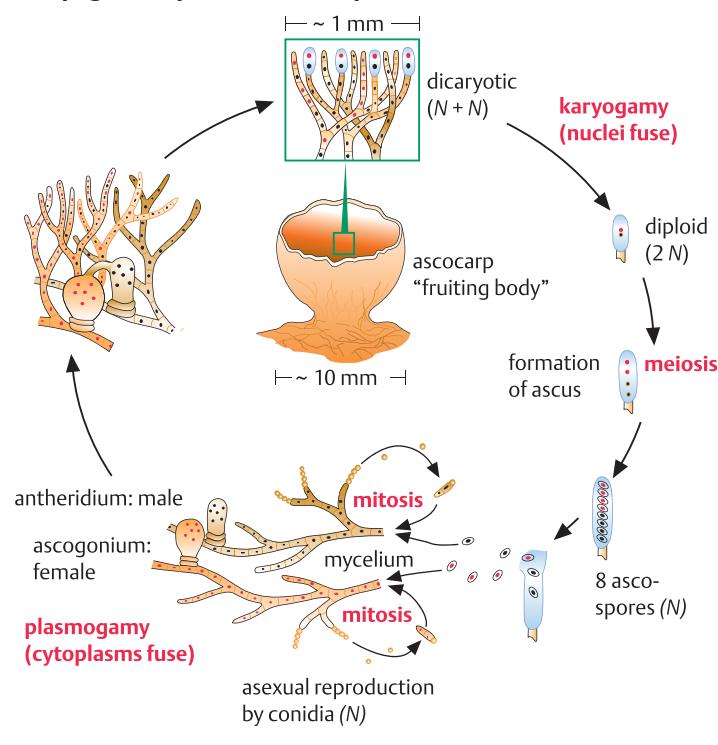
Morphological characteristics of fungi



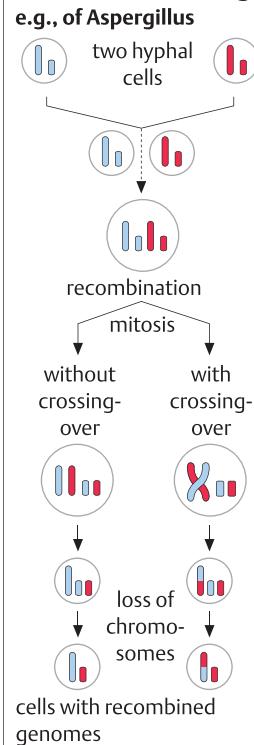
Aspergillus niger, an ascomycete



Propagation cycle of an ascomycete



Parasexual breeding e.g., of *Aspergillus*



Algae

General. Most algae live in water, assimilate CO₂ and produce O₂ through photosynthesis. Unlike terrestrial plants, they do not pass an embryonic stage. Prokaryotic algae are termed cyanobacteria or “blue-green algae” and classified into about 100 genera. Eukaryotic algae form > 20,000 genera and are subdivided into green, brown, red algae, diatoms and others. Some cyanobacteria and algae form toxins (microcystins, saxitoxin), others are used for the production of food additives or specialty chemicals such as, e.g., alginate, agar-agar or astaxanthin. More recently, algae have been explored as a source of bio-energy, since, like terrestrial plants, they use CO₂ as their sole carbon-source, but without competing for agricultural land. Bio-energy may be harvested as algal biomass (formation of biogas) or lipids (triglycerides or isoprenoids), sometimes optimized in yield by metabolic engineering. Cultivation can be performed by aquaculture, in open ponds or in bioreactors. The biotechnology of algae is mainly promoted in nations with plentiful sunshine and long coasts such as the USA, Australia, Japan, Israel and China.

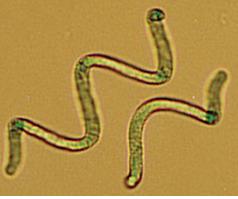
Eukaryotic algae comprise unicellular organisms of some µm size (*Chlorella*), but also large multicellular organisms up to 30 m long (kelp). Algal cells contain compartments such as chloroplasts which, in addition to chlorophyll a and b, often contain carotenoids. Some genera such as, e.g., *Euglena*, can live both as autotrophs or complete heterotrophs. In the latter case, they lose their chloroplasts. The cell wall of many algae is composed of cellulose fibrils which are reinforced by other polysaccharides such as alginic acid. Diatoms form their cell wall from silicates built by silica deposition on a protein matrix. *Laminaria* and other marine brown algae are an important source of alginates (>158). The viscosity of an alginic solution depends on Ca²⁺ concentration. Alginates are used in the food industry as thickening agents, in medicine for surface wound treatments, and recently also as fibers in textile production. *Chlorella* are unicellular freshwater algae which propagate asexually. They contain one chloroplast and only a few mitochondria. Their cultivation is quite simple, and they are used as

food additives. *Botryococcus braunii* is another green freshwater microalgae. Unlike *Chlorella*, it forms colonies. Under appropriate conditions, it can accumulate up to 60 % hydrocarbon content (alkanes, terpenoids, squalen). *Botryococcus* oil is being investigated as a biofuel. *Haematococcus pluvialis* is a freshwater algae which forms cocci. It is able to synthesize the red-colored tetraterpene astaxanthine in high yields. Through the aquatic food chain, astaxanthine is responsible for the reddish color of salmon, shrimps etc.. It is a strong antioxidant which is well tolerated in human nutrition. As a consequence, it is used as a food additive and in cosmetics. *Cryptocodium cibnii* is a marine red algae from the family dinoflagellatae. It can form up to 20 % of its dry mass as dodosahexaenoic acid (DHA), an ω -3-fatty acid (>34, 162), which is used as an antioxidant food additive. *Dunalilla* are halophilic marine microalgae. They form high concentrations of β -carotene and glycerol, the latter as an osmoregulant. *Neochloris oleoabundans* is a green micro-algae which accumulates up to 30 % of its dry mass as triglyceride. This oil is being investigated as a biofuel. The genus *Nannochloropsis* comprises several marine phytoplankton algae, some of which store triglycerides. As they are quite easy to transform, they may have potential for producing alkanes from fatty acids, using synthetic metabolic pathways.

Cyanobacteria are prokaryotic organisms. Some of them can also grow in a heterotrophic manner. They exhibit wide morphological diversity and are divided into 5 classes. Their cell walls are composed of peptidoglycan, and their photosynthetic membranes are multi-layered and complex: besides chlorophyll a, they contain phycobilin pigments. Many cyanobacteria contain “heterocysts” for nitrogen fixation and cyanophycin, an aspartate-arginine copolymer, as a carbon-nitrogen storage compound. The genomes of about 35 cyanobacteria have been sequenced, and the molecular biology of *Synechocystis sp.* is most advanced. *Spirulina* is a 1–3 µm long cyanobacterium which grows in highly alkaline salt lakes. It forms multicellular spiral microfilaments. Spirulina biomass is produced in aquaculture and marketed as a food and feed additive.

Algae

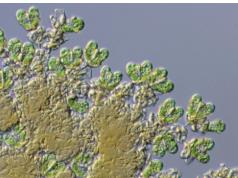
phylogeny	properties	examples relevant for biotechnology
cyano-bacteria	prokaryotes only chlorophyll a cell wall: peptidoglycans	<i>Spirulina</i> , <i>Synechocystis</i> sp.
algae	eukaryotes chlorophyll a and b cell wall: cellulose, polysaccharides, silicate	<i>Euglena gracilis</i> , <i>Chlorella vulgaris</i> , <i>Botryococcus braunii</i> , <i>Haematococcus pluvialis</i> , <i>Dunaliella salina</i> , <i>Nannochloropsis oculata</i> , <i>Neochloris oleoabundans</i> , <i>Laminaria</i>





Spirulina

Euglena




Botryococcus braunii

Chlorella

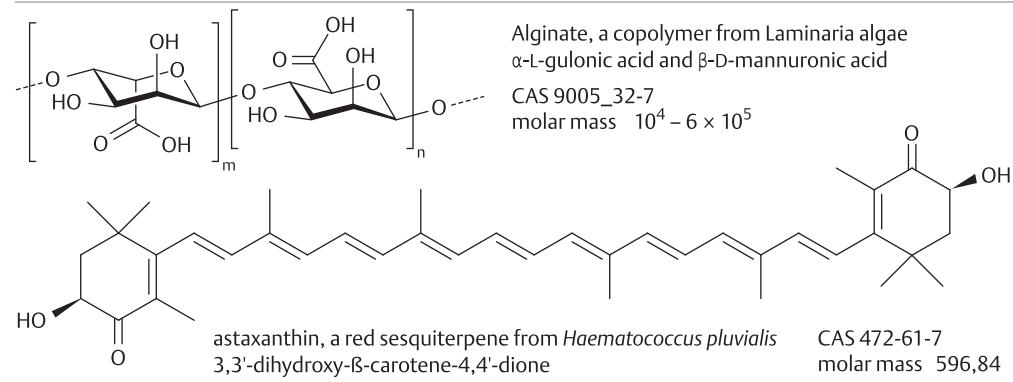
Spirulina platensis cultivation in attached biofilm at CAS Institute for BioEnergy and Bioprocess Technology (QIBEBT), Qingdao, PR China

Industrial manufacture of algal products



Dunaliella salina Farm of Cognis Co., Western Australia

Neochloris oleoabundans biofuel algae farm, conceptual drawing



Some bacteria of importance for biotechnology

General. Some bacteria are especially important in biotechnology. Examples are *Escherichia coli*, *Pseudomonas putida*, *Bacillus subtilis*, *Streptomyces coelicolor*, and *Corynebacterium glutamicum*.

Escherichia coli is a saprophyte in the large intestine of mammals and belongs to the Enterobacteriaceae group. It forms rods that carry flagella. The cell wall stains Gram-negative: it encloses two membranes that include a periplasmic space. Under anaerobic growth conditions, *E. coli* generates energy by fermentation and forms acids. In the presence of O₂, energy is supplied through the respiratory chain. Under optimal conditions, its doubling time is ca. 20 min. The *E. coli* genome is ca. 4.6 Mbp in size, the G+C content is 51 %. Although *E. coli* is among the best understood microorganisms and the genome of *E. coli* K-12 MG1655 was sequenced in 1997, the function of many of its gene products derived from ~4,300 open reading frames (ORFs) is not yet fully understood. In biotechnology, *E. coli* is used as a host organism for the expression of nonglycosylated proteins, e. g., enzymes, insulin, growth hormone, and antibody fragments. Since *E. coli* grows in the human large intestine, it is classified in safety group S2; as a consequence, attenuated *E. coli* strains of reduced genome size are used, in which all risk factors were eliminated and which can be handled under normal microbiological safety conditions as group S1 organisms (e. g., *E. coli* K12) (→332). They are also used for cloning experiments. Various plasmid vectors (→58) have been developed for cloning foreign genes in *E. coli*; for example, the BAC cloning vector is used to construct genomic libraries.

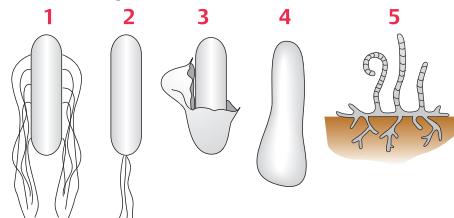
Pseudomonas putida is rod-shaped with polar flagella and lives aerobically in water. The cell wall contains two membranes that enclose a periplasmic space and stains Gram-negative. The *P. putida* genome contains ca. 6.1 Mbp, its G+C content is 61 %. Pseudomonads have a wide genetic potential for the degradation of aromatic compounds, which can be horizontally transferred through plasmids. In biotechnology, they are mostly used in environmental studies (→292). *Bacillus subtilis* is rod-shaped without flagella and lives aerobically in soil.

Under unfavorable conditions, it forms dormant, thermostable spores. Its cell wall stains Gram-positive and encloses only one membrane. Energy is generated via the electron transport chain. Doubling time, under optimal growth conditions, is ca. 20 min. The genome of *B. subtilis* contains ca. 4.2 Mbp and has been completely sequenced; its G+C-content is 44 %. In biotechnology, *B. subtilis* is the preferred microorganism for producing extracellular enzymes, e. g., proteases, cellulases and amylases (→174, 176, 190, 194). It is also used for the production of some antibiotics such as bacitracin. Production strains of 20 % reduced genome size have been engineered which produce up to two-fold more cellulase or protease.

Corynebacterium glutamicum is a member of the coryneform bacteria which grow in many habitats and include some pathogenic species such as *C. diphtheriae*. The club-shaped cells grow aerobically and stain Gram-positive. The *C. glutamicum* genome contains ca. 3.1 Mbp and was completely sequenced in 2003; its GC content is 56 %. Deregulated and metabolically engineered mutants of *C. glutamicum* are important production strains for L-glutamic acid and L-lysine. *C. glutamicum* is a preferred organism for synthetic biology (→320), and mutant strains which overproduce lactic acid (→148), succinic acid (→152), 1,2-propanediol (→142) or aniline from biomass have already been described. A Corynex system based on *C. glutamicum* mutant strains has been proposed for the industrial manufacturing of pharmaceutical proteins in high yields, with excellent down-stream processing.

Streptomyces coelicolor is another soil bacterium from the genus Actinomycetes. It propagates in the form of a mycelium and forms aerial hyphae, from which spore-forming conidia are constructed. The cell wall stains Gram-positive and encloses just one membrane. Like most other Streptomyces strains, *S. coelicolor* degrades cellulose and chitin. Its large linear genome has been completely sequenced and contains ca. 8.7 Mbp, nearly twice the number in *E. coli*; its G+C content is 72 %. The ca. 8,000 structural genes code mainly for enzymes that are required for the formation of secondary metabolites, e. g., for antibiotics (→200).

Some important bacteria in biotechnology



1 *Escherichia coli*

2 *Pseudomonas putida*

3 *Bacillus subtilis*
(germinating from spore)

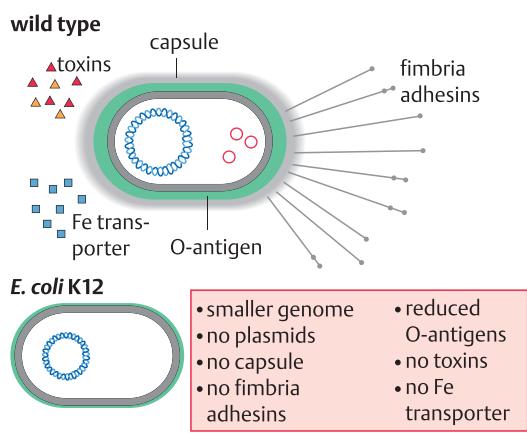
4 *Corynebacterium*
glutamicum

5 *Streptomyces*
coelicolor (with
conidia)

	1	2	3	4	5
flagellation	+	+	-	-	-
Gram-staining	-	-	+	+	+
spore formation	-	-	+	-	+
aerobic growth	+	+	+	+	+
G + C content	51	61	44	56	72
genome size (Mbp)	4,6*	4,2	4,2*	3,1*	8,7*

*genome sequences have been completed

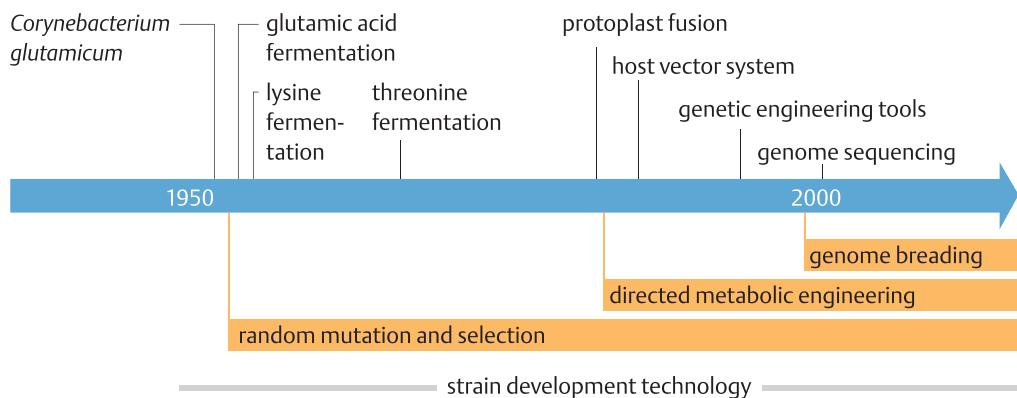
E. coli K12 modifications



E. coli K12 genome: gene functions

total	4485
enzymes	~1500
transport proteins	~600
regulatory proteins	~400
genes of foreign origin	~300
membrane proteins	~250
structural proteins	~200
carrier proteins	~100
RNA synthesis	~150
other	~300
unknown function	~600

Corynebacterium glutamicum



Some completely sequenced genomes of prokaryotes

	disease	genome size (Mbp)
<i>Haemophilus influenzae</i>	childhood meningitis	1.8
<i>Helicobacter pylori</i>	ulcer	1.7
<i>Mycoplasma pneumoniae</i>	bacterial pneumonia	0.8
<i>Mycobacterium tuberculosis</i>	lung tuberculosis	4.4
<i>Treponema pallidum</i>	syphilis	1.1
<i>Mycobacterium leprae</i>	leprosy	3.3

Microorganisms: isolation, preservation, safety

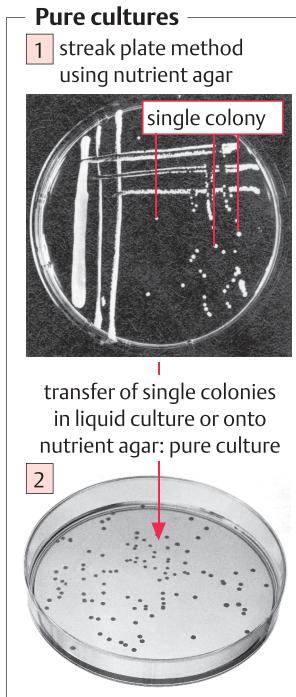
General. For most experiments with microorganisms, pure cultures are used. In biotechnology, most strains have additionally been optimized for a specific application, using rounds of mutation and selection. Microorganisms are maintained and conserved in culture collections. They are propagated on solid or liquid nutrient media under sterile conditions. Most microorganisms used in biotechnology grow aerobically on organic substrates (heterotrophic growth). Photosynthetic microorganisms are cultured under light, anaerobic bacteria under the exclusion of oxygen.

Pure cultures are obtained from culture collections or from their natural habitats (soil, water, food, other organisms) using enrichment cultures. The preferred method for obtaining a pure culture is the streak plate method, in which a mixed culture is spread over the surface of a sterile nutrient agar (a crosslinked polysaccharide isolated from marine algae) with a sterile wire loop (plating). Usually, growth conditions are chosen (→88) that favor the microorganism one wants to isolate (selection) (→24): for example, excluding oxygen and working under light with CO₂ as the sole source of carbon and N₂ as the sole nitrogen source leads to enrichment in cyanobacteria. A sugar medium at slightly acidic pH enriches fungi, incubating at elevated temperatures favors thermotolerant microorganisms, and when casein is the sole nitrogen source, protease-secreting microorganisms have a selective advantage. Based on 16S-rRNA analysis, however, it is believed that < 5 % of all naturally occurring microorganisms can be isolated by these methods (→74).

Culture collections are used to conserve pure cultures. The identity, viability, and metabolic functions of conserved cultures must be tested upon reactivation. The conventional method for conservation consists of transferring a pure culture at regular time intervals to a new agar plate or slant. This method may lead, however, to degeneration. Important type or production strains are therefore preserved under either of the following conditions: 1) under metabolically inert liquids such as mineral oil (suitable for hyphae-forming fungi); 2) freezing at -196 °C in liquid N₂ or at -70 °C

in a deep-freezer; freezing and thawing must be done rapidly and in the presence of glycerol to prevent cell destruction by ice crystals (this method is mainly used for bacteria and yeasts); 3) vacuum drying of cell suspensions on a carrier (sand, silica gel) and in the presence of a mild emulsifier (skim milk, serum) and preservation at -70 °C. In all cases, it must be verified that the conserved strains can be reactivated. Most nations operate large public culture collections from which pure cultures can be ordered. They are either universal for all types of microorganisms (e.g., the American Type Culture Collection, ATCC, or CABRI, Common Access to Biological Resources and Information, a European consortium of general resource collections, e.g., the German *Deutsche Sammlung für Mikroorganismen und Zellkulturen*, DSMZ, and specialized collections for particular groups of microorganisms, such as the Dutch *Centralbureau voor Schimmelcultuuren* CBS). All industrial companies that produce biotechnological products, and many hospitals, have their own culture collections. If the value of a strain lies in plasmid-coded properties (e.g., in the generation of libraries of plasmid-coded enzyme mutants), the preservation of plasmids instead of bacterial strains has become the method of choice. To this end, so-called "plasmid preps" are preserved at -20 °C and can be stored long-term if no nucleases are present. As compared to whole strains, plasmid preps are also simpler to transport or send to other laboratories.

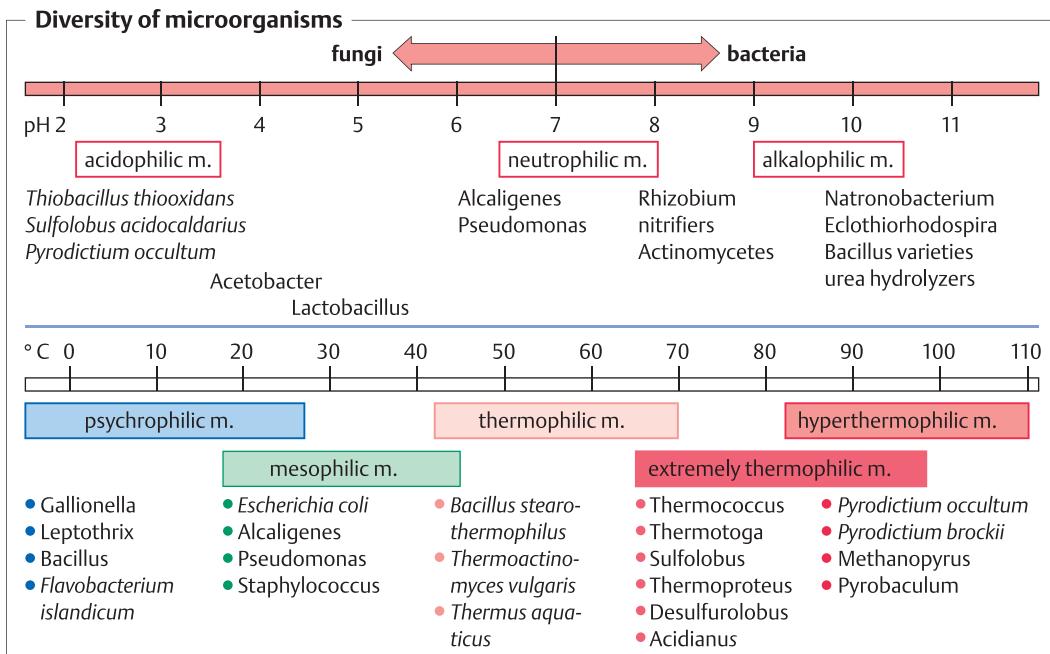
Safety. Each study using microorganisms must comply with biological safety rules (→332), because dangerous pathogens may occur in all microbial isolates (examples: *Bacillus subtilis*: harmless producer of technical enzymes, *Bacillus anthracis*: anthrax pathogen; *Aspergillus oryzae*: used for soy sauce production, *Aspergillus flavus*: forms highly hepatotoxic and carcinogenic aflatoxins). For safety considerations, microorganisms are classified into four risk groups. Both the construction and the equipment of a laboratory and the operating rules must be adapted to the relevant risk group. Risk group 1 (generally safe) includes microorganisms that have been used in food production for centuries, e.g., *Saccharomyces cerevisiae* and *Aspergillus oryzae*. Most microorganisms used in biotechnology fall into risk group 1.



Enrichment cultures (examples)

bacteria	energy source, nutrients
phototrophic	
Rhodospirilla	● light, H ₂ or organic acids, CO ₂
Cyanobacteria	■ light, CO ₂ , N ₂ as nitrogen source
chemolithotrophic	
Nitrosomonas	● NH ₄ ⁺ as H donor, O ₂ as H acceptor
Thiobacillus	● H ₂ S, S or S ₂ O ₃ ²⁻ as H donor
methane formers	■ H ₂ as H donor, CO ₂ as H acceptor
heterotrophic	
Pseudomonads	■ 2 % KNO ₃ as H acceptor, organic acids
Clostridia	■ starch, NH ₄ ⁺ , pasteurized inoculate
Enterobacteria	■ glucose, NH ₄ ⁺
lactic acid bacteria	■ glucose, yeast extract, pH 5
Bacilli	● starch, NH ₄ ⁺
Streptomyces	● mannitol, NH ₄ ⁺
enzyme secretors	
protease-forming strains	● glucose, NH ₄ ⁺ , casein
lipase-forming strains	● glucose, NH ₄ ⁺ , tributyrin

● aerobic or ■ anaerobic growth conditions



Risk groups (selection)

risk group 1	risk group 2	risk group 3
<i>Acetobacter acetii</i> , <i>Agrobacterium tumefaciens</i> , <i>Bacillus subtilis</i> , <i>Lactobacillus casei</i>	<i>Acinetobacter calcoaceticus</i> , <i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i>	<i>Bacillus anthracis</i> , <i>Mycobacterium tuberculosis</i> , <i>Yersinia pestis</i>
<i>Penicillium notatum</i> , <i>Rhizopus oryzae</i> , <i>Aspergillus niger</i> , <i>Candida tropicalis</i>	<i>Aspergillus flavus</i> , <i>Candida albicans</i> , <i>Trichophyton rubrum</i> , <i>Histoplasma capsulatum</i>	<i>Histoplasma capsulatum</i>
bacteria		fungi, yeasts

Microorganisms: strain improvement

General. Microorganisms isolated from environmental samples rarely exhibit all the properties that are required in a technical application. Thus, they are usually optimized by a series of mutation and selection steps. The targets of strain improvement are usually: 1) to increase the yield of the desired product; 2) to remove undesired by-products; and 3) to improve general properties of the microorganism during fermentation (e.g., reduced fermentation time, no interfering pigments formed, resistance to bacteriophages). A great advantage in dealing with microorganisms is their short doubling time (often < 1 h): it allows a very large number of mutants to be produced and screened in a short time. In eukaryotic organisms, e.g., fungi, recombination events must be taken into account. With increasing knowledge of microbial metabolism, its regulation and its coding by the genome, genetic methods that delete or amplify defined metabolic steps in a targeted way are on the increase (metabolic engineering).

Mutation. The spontaneous mutation frequency (changes in DNA sequence due to natural mutation events and errors during replication) is on the order of 10^{-7} for a gene (1,000 bp) of normal stability. Most mutations remain silent or they revert genetically or functionally or by DNA repair mechanisms to the original state. Thus, for industrial strain improvement harsher mutation conditions are required: the use of UV radiation or of mutagenic chemicals are methods of choice, and, depending on the experimental goals, conditions are chosen to achieve a mortality rate of 90 % to > 99 %. Survivors exhibiting the desired properties are then selected according to their phenotypes.

Selection using surface cultures. Phenotype selection is often synonymous with the selective isolation of mutants with high productivity. A key requirement for such experiments is the availability of an indicator reaction. For example, the resistance of a mutant to antibiotics, inhibitors, or phages can be identified if the mutant can grow on a nutrient agar that contains one of these agents. Replica plating first on a nutrient-rich agar, followed by plating on a selection medium, may yield very useful infor-

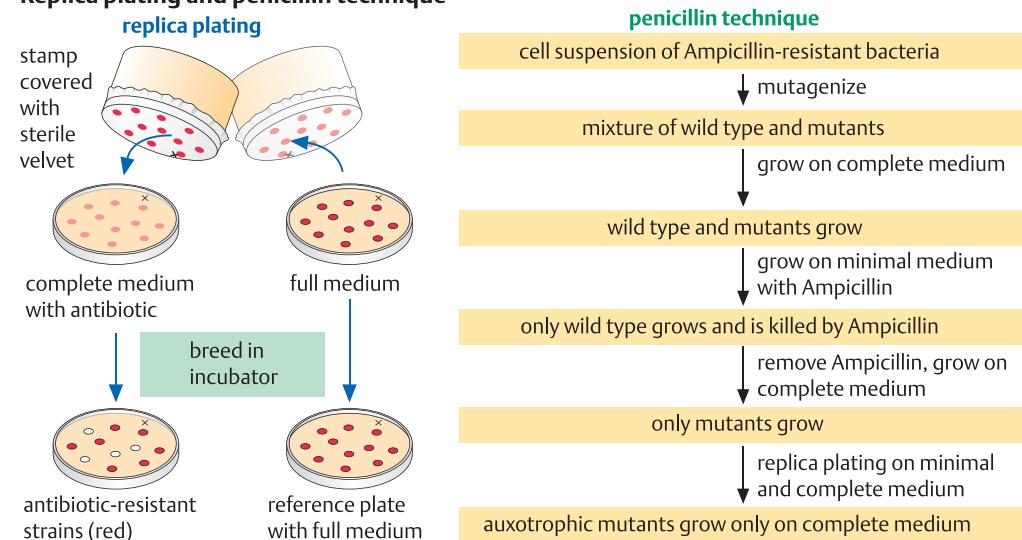
mation. An enrichment step in a penicillin-containing agar (penicillin inhibits only growing cells) can help to identify auxotrophic mutants, which depend on the presence of a given metabolite for growth. If mutants that form a biologically active metabolite (e.g., an antibiotic or an enzyme) in higher yields are to be isolated, the size of inhibition or lysis plaques can be used as an indicator. Thus, if, e.g., lipases are being screened, the diameter of a halo around a clone growing on an agar plate which appears opaque due to its tributyrin content provides a first guess as to the amount of lipase produced. The great advantages of such selection procedures are 1) high flexibility in the choice of the selection criterion and 2) high number of mutants that can be visually screened (several hundred on a single agar plate). If such simple procedures are not available, a high-throughput assay must first be developed. Many procedures have been described to this end. They comprise biochemical indicator reactions, immunoassays or, in the worst case, analysis of each mutant cell, usually distributed into microtiter plates, through HPLC, capillary electrophoresis or similar procedures. Due to the random method of mutagenesis, however, the strains obtained by this kind of selection are usually defective in several genes and must be tested for their robustness as production strains in separate experiments. To this end, they are subjected to further selection with respect to growth, productivity, and other features using shake flasks and then small bioreactors under conditions resembling the production process. The best candidates may then be backcrossed with wild-type or less mutated strains to reduce the negative effects arising from many passages of random mutation.

Selection in submersed culture. Continuous fermentation has also been used for selecting microorganisms. A pure culture of a microorganism is grown in a chemostat in the presence of a mutagenic agent and subjected to selective pressure, e.g., by gradually replacing a good carbon source (→88) with a poor one. During continuous growth, those mutants that are better adapted to the altered growth conditions prevail. This method cannot be used, however, for selecting mutants that form a desired metabolite in higher concentrations.

Strain improvement of microorganisms

mutagens	mechanism	applications
physical agents		
ionizing radiation (x-ray)	leads to single- and double-strand DNA breakage	major genetic alterations
UV light (254 nm)	thymidine and cytidine form dimers	point mutations
chemical agents		
nitrite	deaminates adenine to hypoxanthine, cytidine to uridine	point mutations
alkylating agents	alkylate purines	point mutations
base analogs	are incorporated into replicated DNA	major genetic alterations
acridine orange	intercalates into DNA	major genetic alterations
biological agents		
transposons	transfer DNA elements within a chromosome	gene markers

Replica plating and penicillin technique



Selection media

growth at altered temperature 	minimal medium with metabolite 	medium contains indicator for metabolite 	medium and antimetabolite
temperature mutants 	auxotrophic mutants defective in the biosynthesis of one metabolite* 	catabolic mutants defective in one enzyme of substrate catabolism* 	regulatory mutants altered rate of synthesis of one enzyme or product
medium, test organism, and β lactamase 	medium and test organism 	medium and casein 	medium and tributyrin

* using penicillin technique and replica plating