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Bacterial Gene Transfer by Natural Genetic Transformation in the Environment

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INTRODUCTION

Natural genetic transformation of bacteria encompasses the active uptake by a cell of free (extracellular) DNA (plasmid and chromosomal) and the heritable incorporation of its genetic information. It is a mechanism of horizontal gene transfer and depends on the function of several genes located on the bacterial chromosome. The term "natural genetic transformation" (or natural transformation) has been coined to distinguish it from other (artificial) in vitro procedures used to introduce DNA molecules into bacterial cells. Bacteria are the only organisms capable of natural transformation. It can be considered the genuine bacterial gene transfer process since other gene transfer processes are determined by genes located on plasmids and transposons (conjugation) and on bacteriophages (transduction).

Natural transformation of bacteria was detected more than five decades ago in laboratory experiments (for a historical review, see reference 343). Several independent lines of research have provided convincing evidence that transformation occurs in the environment. One source of data is the examination of the transformation process itself. The results of in vitro studies on the transformation of many bacterial species and strains, the evidence for the existence of extracellular DNA in the environment, the demonstration of the availability of that DNA for uptake in bacteria, and the experiments showing that bacteria can develop the physiological state of competence for DNA uptake under conditions simulating those of natural bacterial habitats are all consistent with a bacterial gene transfer by free DNA occurring in the environment. These topics will be considered in this review. The other source of evidence is the analysis of nucleotide sequences of homologous genes from many species which may lead to the finding of identical genes or identical parts of genes among evolutionarily unrelated organisms. The existence of mosaic genes and chromosomes, also evidenced by enzyme pattern analysis that indicates allelic variation in multiple chromosomal genes, can be explained as the result of horizontal gene transfer, in several instances particularly by transformation. Results of such retrospective studies will also be summarized.

Ecological Requirements of Bacterial Gene Transfer Processes

The specific requirements of each of the three gene transfer mechanisms, transformation, conjugation and transduction, suggest different probabilities for their occurrence in the various natural habitats. Conjugation has the greatest requirements. The donor cell must contain a conjugative element (plasmid or transposon), and donor and recipient cells must establish a physical contact sufficiently stable to allow transfer of DNA. Coincidentally, both cells have to be metabolically active to allow DNA synthesis and other activities (145). Gene transfer by transduction requires a metabolically active donor cell in which transducing phage particles are produced during viral reproduction. The recipient can be spatially and temporarily separated from the donor, because the genetic information in the transducing particle can persist. Phages are often resistant to many physical and chemical agents and can survive in the environment particularly when adsorbed on clay minerals and other particulates (351). The recipient must be related to the donor by common sensitivity to the bacteriophage, but extensive metabolic activity is not required for the infection process (171). Gene transfer by transformation does not require even a living donor cell, because release of DNA during death and cell lysis suffices to provide free DNA (see the section on release of DNA from cultured cells, below). The persistence and dissemination of DNA in the environment determine how far in time and space the recipient cell can be separated from the donor (185). The recipient must be physiologically active to be able to take up DNA (see the section on biology of natural genetic transformation, below). A close genetic relationship between donor and recipient cells is not necessary for transformation with plasmid DNA (see the section on reconstitution of plasmid molecules, below). Thus, natural transformation has several features enabling it to occur even in populations and communities that experience extreme environmental changes or encounter great fluctuations of population dynamics.

Natural and Other Bacterial Transformation

The natural transformability of a limited number of bacterial species has been known for some time and has been used to introduce DNA into cells. Since the widespread application of recombinant DNA techniques, other methods for the introduction of genetic material into cells (prokaryotic and eukaryotic) have been developed. They are also termed transformation (sometimes transformation after artificially induced competence or just artificial transformation) and are applied in many more laboratories than those examining natural transformation. The various methods used to make duplex DNA translocate into cells and their variations (232) can be grouped into the following principal procedures: (i) treatment of cells with solutions of $CaCl_2$ or chlorides of other elements including Mg, Ba, Rb, Sr, and mixtures of them; (ii) treatment of cells with chelating agents (e.g., EDTA); (iii) treatment of cells with enzymes (muraminidases or peptidases), leading to the formation of spheroplasts or protoplasts; (iv) fusion of cells or protoplasts with DNA, with cells or with DNA packaged in liposomes (often with the aid of polyethylene glycol); (v) freezing and thawing of cells; (vi) exposure of cells to electric fields (electroporation); and (vii) bombardment of cells with small particles, transporting DNA into the cytoplasm (biolistic transformation).

One can imagine that bacteria in the environment may encounter situations which are similar to the conditions of the above procedures. For instance, a decrease in temperature below the freezing point of water, the presence of solutions of certain electrolytes, and the presence of lysozymes and proteolytic enzymes are situations likely to be found in aquatic and terrestrial environments or within higher organisms. As an example, mixing *Escherichia coli* cells with the supernatant of a culture of a plasmid-bearing strain and freezing and thawing the mixture can give rise to transformants (187, 361). The fact that high concentrations of protoplast cells are present in certain habitats (e.g., *Mycoplasma* spp., bacteroid *Rhizobium* spp. within the nodules of leguminous plant roots) would also argue that spontaneous DNA transfer (artificial transformation?) can occur in natural bacterial communities. In contrast, natural transformation depends on a set of cellular functions provided by genes dispersed over the chromosome (79, 123) and coordinately expressed under the influence of particular environmental conditions. This may lead one to suspect that cells actively respond to conditions in the habitat by adjusting their level of gene acquisition through natural transformation rather than being passively subjected to environmentally enforced gene exchange (see the section on competence development under environmental conditions, below).

Gene Transfer Processes in the Environment

The horizontal transfer of genetic material among bacteria in microbial ecosystems has gained much attention since the debate about the potential risks conferred upon the environment by the accidental or deliberate release of genetically engineered organisms. The ecosystems considered in the relevant studies were from the aquatic (wastewater, freshwater, seawater) and terrestrial (sediments, soils) environment. The genetic ecology of bacteria, including physiology, molecular genetics, and population biology of prokaryotes in the various environments, has developed into a new research area at the border of biological and environmental sciences. Studies of bacterial gene transfer by conjugation (for a review, see reference 145) indicated that an extensive gene exchange network may exist between bacteria and even between bacteria and fungi, plants, and animals (for a review, see reference 131). The gene exchange network among bacteria probably also relies on transduction and transformation and is assumed to have an important impact on the dynamics of bacterial communities and ecosystems (205). Several books and special reviews that concentrate mainly on conjugation or transduction in the environment have been published in the last few years (25, 58, 170, 171, 186, 205, 291, 311, 350, 352, 353, 369). The present review will introduce the reader to natural transformation with emphasis on the bacteria living in the environment and on the influence of their habitat characteristics on gene exchange via free DNA. It is not the intention of this review to give a fully detailed compilation of the biology of transformation and the regulation of competence genes, although general aspects are discussed. Details may be found in a number of excellent overviews (79, 80, 251, 325, 343, 344). The aim of this review is to give an overview of the research concepts, experimental strategies, and findings which relate to the bacterial gene flux by natural genetic transformation in several bacterial habitats, especially soil, sediment, and water.

For a systematic experimental investigation, it was previously proposed (384) to dissect the complex process of gene transfer by free DNA into separate steps and to examine each of them with the appropriate strategies and techniques in environmental simulations. The major steps are (i) the release of DNA from cells, (ii) the dispersal and (iii) the persistence of the DNA in the environment, (iv) the development of competence for DNA uptake by cells in the habitat, (v) the interaction of cells with DNA and the uptake of DNA, and (vi) the expression of an acquired trait following DNA uptake. As this concept has proven successful for the identification and analysis of environmental factors influencing natural transformation, we have followed its principle through large parts of this review.

BIOLOGY OF NATURAL GENETIC TRANSFORMATION

Natural transformation (hereafter simply called transformation) differs from conjugation and transduction by the in vitro sensitivity of the process to DNases, because the transfer of genes occurs via free DNA. Genetic competence is defined as the ability of a cell to take up free DNA from the surrounding medium. During growth, in several instances under specific conditions (the so-called competence regime), cells develop the capability to bind and take up DNA. Competence development depends on the expression of genes, whose proteins provide the necessary functions. Table 1 summarizes the presently known bacterial species which were shown to develop natural competence. Apparently, this property is widely distributed among the taxonomic and trophic groups (including archaebacteria), which infers a long evolutionary history of natural competence. It should be noted that inclusion of a strain in Table 1 required that the competence regime be identified. It is expected that many more species develop competence. The following sections will discuss the development of competence of various organisms as well as the process of transformation itself.

Development of Competence

In most naturally transformable bacteria competence is transient. Only in *Neisseria gonorrhoeae* is competence constitutive (331). In the other transformable bacteria it is an inducible physiological property. In *Haemophilus influenzae*, the development of competence starts when the cells are transferred to defined media which do not allow growth or when cell division is blocked under conditions permissive for

TABLE 1. Naturally transformable prokaryotic species[a]

Species isolated from terrestrial or aquatic habitats	Transformation frequency (chromosomal marker transformants/viable cell)	Reference(s)
Photolithotrophic		
Agmenellum quadruplicatum	4.3×10^{-4}	342
Anacystis nidulans	8.0×10^{-4}	320
Chlorobium limicola	1.0×10^{-5}	258
Nostoc muscorum	1.2×10^{-3}	368
Synechocystis sp. strain 6803	5.0×10^{-4}	115
Synechocystis sp. strain OL50	2.0×10^{-4}	206
Chemolithotrophic		
Thiobacillus thioparus	10^{-3}–10^{-2}	394
Thiobacillus sp. strain Y	1.7×10^{-3}	394
Heterotrophic		
Achromobacter spp.	+[b]	156
Acinetobacter calcoaceticus	7.0×10^{-3}	158
Azotobacter vinelandii	9.5×10^{-2}	263
Bacillus subtilis	3.5×10^{-2}	246
Bacillus licheniformis	1.2×10^{-2}	102
Deinococcus (Micrococcus) radiodurans	2.1×10^{-2}	365
Lactobacillus lactis	2.3×10^{-5}	138
Mycobacterium smegmatis	10^{-7}–10^{-6}	250
Pseudomonas stutzeri (and related species)	7.0×10^{-5}	38
Rhizobium meliloti	7.0×10^{-4}	59
Streptomyces spp.	+[c]	299
Thermoactinomyces vulgaris	2.7×10^{-3}	141
Thermus thermophilus	1.0×10^{-2}	173a
Thermus flavus	8.8×10^{-3}	173a
Thermus caldophilus	2.7×10^{-3}	173a
Thermus aquaticus	6.4×10^{-4}	173a
Vibrio sp. strain DI9	2.0×10^{-7}	149
Vibrio sp. strain WJT-1C[d]	2.5×10^{-4}[e]	93
Vibrio parahaemolyticus	1.9×10^{-9}[e]	93
Methylotrophic		
Methylobacterium organophilum	5.3×10^{-3}	255
Archaebacteria		
Methanobacterium thermoautotrophicum	+[b]	393
Methanococcus voltae	8.0×10^{-6}	22
Clinical isolates of pathogenic species		
Campylobacter jejuni	2.0×10^{-4}	386
Campylobacter coli	1.2×10^{-3}	386
Haemophilus influenzae	7.0×10^{-3}	218
Haemophilus parainfluenzae	8.6×10^{-3}	118
Helicobacter pylori	5.0×10^{-4}	122
Moraxella spp.	+[b]	154–157
Neisseria gonorrhoeae	1.0×10^{-4}	218
Neisseria meningitidis	1.1×10^{-2}	41
Staphylococcus aureus	5.5×10^{-6}	306
Streptococcus pneumoniae	2.9×10^{-2}	178
Streptococcus sanguis	2.0×10^{-2}	19
Streptococcus mutans	7.0×10^{-4}	315

[a] Modified from reference 200.
[b] Qualitative determination (streaking on selective medium following plate transformation [158]).
[c] Measurement of uptake of tritium-labeled DNA.
[d] A high-frequency-of-transformation mutant of DI9.
[e] Plasmid-encoded antibiotic resistance marker.

protein synthesis (325). Competence develops as cells begin to grow and reaches its maximum during early to late log phase in *Acinetobacter calcoaceticus* (272), *Azotobacter vinelandii* (266), *Staphylococcus aureus* (306), *Streptococcus pneumoniae* (325), and *Anacystis nidulans* R2 (43) or during the transition from log phase to stationary phase as observed in *Bacillus subtilis* (325), *B. stearothermophilus* (reviewed in reference 150), *Chlorobium limicola* (258), *Methylobacterium organophilum* (255), *Pseudomonas stutzeri* (38, 201), *Synechocystis* spp. (115, 206), and *Vibrio* sp. (94). In *Agmenellum quadruplicatum* (341), *Deinococcus* (*Micrococcus*) *radiodurans*, and *Mycobacterium smegmatis* (250, 365) competence proceeds throughout exponential growth and declines during the stationary phase. The portion of cells able to take up DNA in a culture can be estimated from the transformation frequency obtained, e.g., with an auxotrophic marker on homologous chromosomal DNA, considering that the marker is present on only 1 of approximately 100 to 200 DNA fragments per chromosome. The competent fraction of a population may make up only a small percentage of the culture, as was found with *P. stutzeri* (203) and with *Streptomyces virginiae* and *Streptomyces kasugaensis* (299), or between 10 and 25%, as in *B. subtilis* (325) and *Acinetobacter calcoaceticus* (271), or almost 100% as in *Azotobacter vinelandii* (73, 99) and in *H. influenzae* and *S. pneumoniae* (325). At a critical cell concentration, competence development in *S. pneumoniae* and *B. subtilis* is induced by an excreted small polypeptide called competence factor (325). The deduced amino acid sequence of the ComA protein of *S. pneumoniae* is very similar to that of the *Escherichia coli* hemolysin protein (HlyB) and to that of ATP-dependent transport proteins of various bacterial species (143). Possibly ComA mediates the transport of the pneumococcal competence factor across the cytoplasmic membrane (143). The most intensively studied naturally transformable species with respect to the genetics and regulation of competence is *B. subtilis*. Several regulatory gene products are involved in the control of postexponential expression of functions not only for competence but also for sporulation, production of antibiotics and degradative enzymes, and motility. The complex regulatory pathways for competence development are beyond the scope of this review, and the interested reader is referred to two recent comprehensive reviews (79, 80). Recently, the *com101A* locus, essential for transformation of *H. influenzae* (182), has been shown to be transiently expressed during competence development (181). Aspects of the environmental regulation of competence in various transformable bacteria will be discussed later in this review (see the section on competence development under environmental conditions).

Streptococcus-Bacillus and *Haemophilus-Neisseria* Models of Transformation

Several stages of transformation can be distinguished, such as the binding of DNA, its processing and transport into the cytoplasm, and the processes necessary for the propagation of the DNA.

In the past, two main DNA uptake routes have been described. Characterization of the processes involved have led to the *Streptococcus-Bacillus* model (gram-positive bacteria) and the *Haemophilus-Neisseria* model (gram-negative bacteria) of transformation (325). In the following, the major steps will be compared among organisms of each model group in some detail. From the recent examination of further species capable of transformation (see the following sections), it appears that separation into the two classic models is artificial because some of these bacteria share features of one model with typical

features of the other. For references on details of the transformation of *B. subtilis*, *S. pneumoniae*, and *H. influenzae*, the reader is referred to the previous reviews on the topic (79, 80, 104, 251, 325, 343, 344). Pertinent studies more recently published will be considered.

DNA binding. In *B. subtilis* and *S. pneumoniae*, double-stranded DNA (but not glucosylated DNA or RNA) associates rapidly with competent cells to form a complex that is resistant to gentle washing or to replacement by excess DNA but sensitive to DNase treatment. At acidic pH, *B. subtilis* also binds and takes up single-stranded DNA (325). In competition studies, it was found that double-stranded DNA of any source (*E. coli*, phage T7, plasmids) adsorbs to the cell surface and is taken up, indicating no specificity of this process for homologous DNA. About 50 (*B. subtilis*) and 30 to 80 (*S. pneumoniae*) DNA binding sites per competent cell, respectively, seem to be involved in the noncovalent association (79, 325).

H. influenzae can bind and take up double-stranded and single-stranded DNA (287), the latter at a low pH of 4.4 (104). In other aspects the transformation system of *H. influenzae* is different from that of *B. subtilis* and pneumococci. This organism binds and takes up double-stranded DNA only from the same or closely related species. This specificity for homologous DNA results from the recognition by the DNA receptor protein of an 11-bp sequence dispersed on the *H. influenzae* chromosome in about 600 copies (mean distance, about 4 kb [63, 324]). Of the at least 12 recognition sites on the *H. influenzae* phage HP1*c*1 genome (34.4 kb), 3 were found to contain only the first 9 nucleotides, which were shown in other experiments to be essential for the high-affinity binding/uptake system (91). In addition to the presence of a recognition site, the A+T content of the flanking DNA region also influences the binding and uptake of a DNA molecule. A DNA molecule with A+T-rich regions flanking the recognition site was taken up 48 times more frequently than was a molecule with G+C-rich regions (63). At low pH heterologous DNA is bound, suggesting that nonspecific uptake also occurs (104). During competence development *H. influenzae* synthesizes a set of polypeptides which, in addition to two log-phase proteins, are located in the cell envelope of exclusively competent cells (400, 401). One mutant (the *com-58* mutant) is deficient for DNA-binding activity. The mutant lacks a periplasmic protein which could be the receptor for reversible, high-salt-wash-sensitive adsorption of DNA to the cell surface preceding tight binding and uptake (69, 104). About four to eight sites for DNA binding and uptake are present per cell (69).

The interaction between DNA and a competent cell seems to be quite similar in *N. gonorrhoeae* and *H. influenzae*. Transformation of both species is not inhibited by heterologous DNA (110, 218). In *N. gonorrhoeae*, a 10-bp sequence is recognized by the cell surface binding/uptake system (105). This sequence, which is not recognized by *H. influenzae* (218), is arranged as inverted repeats and forms part of the transcriptional terminators (105). *N. gonorrhoeae* is transformable by single-stranded and double-stranded DNA with similar efficiency and without the need for a downshift of the pH (338).

DNA processing and uptake. Shortly after binding to *S. pneumoniae* cells, single-strand breaks, about 6 kb apart, are introduced into the DNA by an endonuclease activity (EndA) of the cell surface DNA receptor (325). Uptake is defined as the transition of bound DNA into a DNase-resistant state. It is not yet clear whether this occurs by movement of DNA into a vesicle, into the periplasmic space, or directly into the cytoplasm. The transition is initiated by the generation of double-strand breaks, presumably through cleavage at an opposite site near the initially introduced nick. Fragmented double-stranded DNA associated with the cell and still in a DNase-sensitive state participates in the entry into the cell. After uptake, DNA is recovered from inside the cell as single-stranded material. This material is said to be in an eclipse state because upon reextraction from cells it has no transforming activity (325). Uptake requires Ca^{2+}. Recent investigations showed that entry is polar, starting at the 3′ end of the nicks introduced during binding (230). Concomitantly with entry, an amount of labeled acid-soluble material, equivalent to that of acid-precipitable material taken up, is released into the medium, indicating degradation of the strand complementary to the entering strand. By using 3′- or 5′-end-labeled DNA molecules, Mejean and Claverys (231) found a 5′-to-3′ polarity of degradation, which is opposite to the polarity of entry. The rate of entry at 31°C was estimated as approximately 100 nucleotides s^{-1} (231). During translocation into the cytoplasm, the single strand becomes complexed with a protein that has a molecular weight of 19,500. One of the functions of this protein may be the protection of the single strand against nucleases; another function may be to facilitate recombination (see the next section).

The processes that lead to the uptake of bound DNA in *B. subtilis* parallel those in *S. pneumoniae* (79). Double-strand breaks are introduced into the bound DNA, whereas single-strand breaks are not detectable. Uptake is reported to depend on Mg^{2+} on the basis of the finding that entry of [^{3}H]DNA was impaired when Mg^{2+} was omitted from the mineral medium or complexed by EDTA (96). Further, when the concentration was raised from 0.1 to 10 mM Mg^{2+}, the number of *B. subtilis* transformants increased more than 100-fold. Recent investigations with competent cells of *B. subtilis*, which were washed and suspended in Tris-HCl buffer with one of several tested cations, showed that transformation was enhanced not only by Mg^{2+} in a concentration-dependent fashion but also by Na^+, K^+, or NH_4^+ (0.5 to 100 mM). A combination of 5 mM Mg^{2+} and 100 mM Na^+ exceeded the maximum levels of transformation observed with a single cation by two- to threefold (302). Apparently, one step of DNA uptake requires monovalent cations at rather high concentrations. Since the translocation across the cytoplasmic membrane depends on Mg^{2+} (33, 96), the monovalent cations may be required for some step before uptake, possibly for the binding of DNA in a DNase-resistant state. The use of uncoupling agents has revealed that uptake is an energy-dependent process, driven by the electrochemical gradient. Several models which have been proposed to explain the energetics of the DNA transport in *B. subtilis* are discussed by Dubnau (79). One model, though not explaining the requirement for a nuclease, proposes that DNA passes through an aqueous channel into the cell. The deduced amino acid sequences of certain Com proteins of *B. subtilis* show similarities to the primary structure of various proteins of other bacteria (reviewed in reference 79). These proteins form components of the pullulanase secretion system in *Klebsiella pneumoniae* and a protein of the *virB* operon whose functions are thought to promote the transport of T-DNA from *Agrobacterium tumefaciens* to plant cells. Further, the amino acid sequences of certain Com proteins are similar to those of pilin and proteins required for the processing and assembly of pilin into the pilus structure of *Pseudomonas aeruginosa*. The striking similarity of the assembly/export systems and the *com* system of *B. subtilis* suggests a direct or morphogenetic role of late *com* products in the transport of DNA into the cytoplasm (79).

There is a marked difference between the *S. pneumoniae-B. subtilis* and the *Haemophilus* systems regarding the steps following the first reversible association of DNA with cell

surface structures. As mentioned in the previous section, specifically homologous DNA is bound in a salt wash-sensitive and DNase-sensitive state by *H. influenzae*. Subsequently, DNA is rapidly turned into a DNase-resistant stage without apparent production of equivalent amounts of acid-soluble material as described for the gram-positive species. During competence development, membrane vesicles with DNA-binding capacity are formed in *H. influenzae* and *H. parainfluenzae*; these vesicles are visible in the electron microscope. Evidence that these structures are DNA uptake sites has come from studies with transformation-deficient mutants. In *H. influenzae com-51* and *H. parainfluenzae com-10* mutants, vesicles shed into the medium had DNA-binding activities for chromosomal *Haemophilus* DNA (104). Competent wild-type *H. influenzae* cells release the vesicles only when they lose their competence, e.g., when transferred into a growth medium. The vesicles, called transformasomes, extend about 35 nm from the cells, are 20 nm in diameter, and are located on the cell surface where the inner and outer membranes appear to be fused (159). On the basis of DNA uptake studies, Kahn et al. (159) developed the following model. Homologous DNA is taken up as a double strand in an unknown way into the transformasome, where the DNA is protected against exogenous DNase. In this state the DNA is also protected against restriction enzymes and other nucleolytic enzymes of the cell. After a lag of <5 min, linear DNA is transported into the cytoplasm. Since considerable degradation of DNA accompanied the exit from the transformasome and donor label was found randomly incorporated in the chromosome, the model proposes that the DNA molecule is transported as a single strand while the other strand is degraded. Transformation requires 1 mM Ca^{2+} and 100 mM Na^{+} (325). Covalently closed superhelical and relaxed DNA of plasmids containing the 11-bp recognition sequence were also efficiently taken up into the transformasomes. However, superhelical and some relaxed molecules were not further transported into the cytoplasm (159), which may explain the low efficiency of plasmid transformation observed in *H. influenzae* (119). Possibly the supercoiled structure of plasmids or the absence of free ends prevents the translocation of the DNA from the transformasome into the cytoplasm (159).

Integration of chromosomal donor DNA into the recipient chromosome. Integration into the recipient chromosome seems to be similar in the *Streptococcus-Bacillus* and *Haemophilus-Neisseria* types of transformation. After the single-stranded chromosomal donor molecules have entered the cytoplasm, they can be integrated in homologous regions of the recipient chromosome. The heteroduplex is thought to be formed by the assimilation of the donor strand concomitant with the displacement of the corresponding recipient strand. In *B. subtilis*, about 70% of the single-stranded homologous DNA taken up into the cytoplasm is integrated (79). The recipient chromosome seems to be prepared for the integration of donor DNA. An enhanced level of single-stranded gaps was found in competent cells of *B. subtilis* (126) and *H. influenzae* (226). The size of the integrated donor strands in *B. subtilis* varies but seems to average 8.5 kb (79, 325). The formation of the donor-recipient complex is presumably catalyzed by a RecA-like function. The assumed role of this general recombination protein in heteroduplex formation is substantiated by the facts that *B. subtilis recA* (formerly called *recE*; the *E. coli recA* analog) mutants are transformation deficient, apparently because of the lack of heteroduplex formation (81). Further, the level of RecA protein in competent *B. subtilis* cells is increased about 10-fold over that in noncompetent cells. The competence-specific induction of the RecA protein seems to be independent of RecA function (208). This is in contrast to the SOS system of *E. coli*. Here a family of genes (*din*) are induced coordinately, following DNA damage, by a mechanism involving activation of the coprotease activity of RecA, which facilitates the autocatalytic cleavage and thereby inactivation of the LexA protein, the common repressor for all *din* operons (for reviews, see references 236 and 385). The DNA damage-independent derepression of *rec* genes would supply the competent cell with a high recombination potential for efficient integration of donor DNA. Before replication, mismatches in the heteroduplex can be repaired with variable efficiencies, depending on the nature and extension of heterology. This mismatch correction system, known as Hex in *S. pneumoniae*, will be dealt with below (see the section on transformation in the environment).

Reconstitution of plasmid DNA molecules. If the donor DNA in transformation is a plasmid (or phage DNA), it is processed and transported like chromosomal DNA, leading to fragmentation of the molecule (19, 54, 72, 159, 286, 308). Generally, transformation efficiencies (number of transformants per microgram of DNA) by plasmid DNA are lower than by chromosomal DNA, although plasmid DNA is enriched for the selected marker compared with chromosomal DNA. Isolated monomer plasmid DNA transformed *S. pneumoniae* with a second-order dependence on DNA concentration, whereas chromosomal DNA and dimer plasmid DNA gave a single-hit response (309). As described above, internalized chromosomal single-stranded fragments are rescued by a recombination process with the chromosome. Accordingly, Saunders and Guild (309) interpreted the two-hit dependence of monomer plasmid transformation to mean that a separate entry of two complementary linear plasmid single strands had to occur. These can hybridize and will form a circular molecule when the double-strand breaks preceding single-strand uptake were not at identical positions on the plasmids. The circular molecule will have gaps if the entire complementary single strands were not taken up. Repair synthesis can restore an intact replicon. Further experimental support for this mechanism has come from studies with *Streptococcus sanguis* (19). In these experiments, linearized plasmids (produced by cleavage with a restriction endonuclease) were found to be inactive in transformation unless a mixture of molecules that had been cut with different restriction enzymes was used. Also, simultaneous uptake of a small and a larger plasmid sharing a region of homology and linearized with different restriction enzymes gave transformants which contained the small plasmid. Apparently, the association of the single-stranded material of the two plasmids had resulted in the loss of the heterologous (presumably unpaired) region of the large plasmid. Furthermore, transformation with oligomeric plasmid DNA may lead to the reconstitution of an intact duplex monomer when a more-than-unit-length molecule and a shorter fragment of the opposite strand simultaneously enter the cell. According to this model, the short fragment would serve as a primer for DNA synthesis, allowing circularization by an intramolecular recombination event between the single-stranded and double-stranded regions at the ends of the molecule (309). An alternative model proposes that the initiation of synthesis of the complementary strand occurs at *ori* V of the plasmid, followed by circularization and restoration of the replicon as described above. Canosi et al. (37) suggested two pathways of plasmid transformation in *B. subtilis*. One pathway of plasmid rescue requires a region of homology of the donor plasmid molecule with the recipient molecule. The homology can be provided by an insert of *B. subtilis* chromosomal DNA in the plasmid. A prerequisite of transformation via this route is that cleavage of the donor plasmid during uptake takes place within the homologous

sequence. It is assumed that the homologous sequences flanking the break in the single-stranded donor molecule synapse with the chromosome. DNA synthesis and ligation convert the linear single-stranded plasmid DNA into a circular molecule, to which the second strand is synthesized. It then can replicate. The function of the *recA* gene is essential for this homology-facilitated plasmid transformation (37), which, like chromosomal transformation, shows first-order dependence on DNA concentration (see, e.g., reference 387). This homology-facilitated pathway of plasmid rescue seems to hold true for *S. pneumoniae* and *H. influenzae* also (191, 355). The other (minor) plasmid transformation pathway in *B. subtilis* is not dependent on homology to resident DNA, and no functional RecA protein is needed. Complementary, overlapping strands are proposed to hybridize and thereby circularize, which would be similar to the *Streptococcus* model (37, 72, 234). Unlike the situation in streptococci, transformation of *B. subtilis* by monomeric plasmids (without a chromosomal insert) is almost not detectable (about 4 orders of magnitude less frequent than transformation by oligomeric forms [244]). This inefficient transformation by monomers seems to be unique to *B. subtilis*. Perhaps entry of donor molecules from different uptake sites in conjunction with a low stability of the single-stranded material in the cell greatly reduces the frequency with which two strands meet and hybridize (37).

Types of Transformation in Other Naturally Competent Bacterial Species

The difference in DNA binding and DNA processing has led, in the past, to the conclusion that transformation in gram-positive (*B. subtilis*, *S. pneumoniae*) and gram-negative (*H. influenzae*, *N. gonorrhoeae*) bacteria are distinct processes (325). However, the data discussed in the following sections indicate that the two types of transformation have many features in common, such that the two DNA uptake machineries are rather like modifications of a general transformation system. Future comparisons of the nucleotide sequences of *com* genes from different organisms will perhaps reveal a relatedness of the genes, as has been shown for two different competence genes in comparisons between *B. subtilis* and *H. influenzae* (123a). In the following sections we have summarized the knowledge of DNA uptake processes of other naturally transformable bacteria.

Azotobacter vinelandii. A thorough physiological characterization of transformation has been done with naturally competent gram-negative bacterium *Azotobacter vinelandii* (73, 74). Doran and Page (74) made use of the heat sensitivity of competence to monitor the fate of donor DNA during uptake. They were able to show that the transition of DNA across the cytoplasmic membrane was blocked in cells incubated at 42°C. The ability to bind [^{32}P]DNA in a DNase-resistant state was not affected by heat, suggesting that transport of adsorbed donor DNA into a protected state in the periplasm occurred prior to entry into the cytoplasm. Plasmid DNA appeared to be taken up by the same mechanism (73). The Mg^{2+}-dependent, DNase-resistant binding event was rapid, being detectable 2 s after contact of cells with transforming DNA. It was specific for double-stranded DNA and was not accompanied by the release of an equivalent amount of acid-soluble material into the medium, which is a characteristic of the *Haemophilus* transformation system (74). DNase-resistant DNA binding was not a competence-specific property, since cells of competent and noncompetent cultures bound equivalent amounts of [^{32}P] DNA. Perhaps the DNA entry machinery is built up during competence development. A notable difference from *Haemophilus* and *Neisseria* spp. was the ability of competent cells, after preincubation with transforming DNA, to bind a second DNA species in a DNase-resistant state. Although homologous, this protected second DNA species could not transform the cells. Doran and Page (74) suggested two types of receptors for DNase-resistant DNA binding. According to their hypothesis, one receptor would bind DNA nonspecifically and the other would bind only homologous DNA as *H. influenzae* and *N. gonorrhoeae* do. The validity of this hypothesis needs further support from results of genetic analysis. The assumed existence of two types of DNA receptors would also explain the data of DNA competition studies showing that nontransforming homologous DNA had a higher tendency for blocking transformation than did heterologous chromosomal or plasmid DNA (73, 74).

Pseudomonas stutzeri. Transformation of *P. stutzeri* exhibits selective DNA binding and uptake properties (32, 38, 201). However, selectivity is not as strict as in *Haemophilus* and *Neisseria* spp. In *P. stutzeri*, the transformation frequency by an auxotrophic marker on homologous DNA decreased to 78% in the presence of heterologous linear duplex DNA (*E. coli*, salmon testes DNA) at a 10-fold mass excess (201). DNA-binding assays (using transformation as the detection system after removal of DNA from cells by centrifugation or DNase treatment) indicated that DNA adsorption to the cells was rate-limiting for transformation. In contrast, a 10-fold excess of heterologous DNA did not reduce the transformation frequency of *H. influenzae* and *N. gonorrhoeae* by a chromosomal streptomycin resistance marker (218). It is not yet known whether a recognition sequence causes DNA uptake specificity in *P. stutzeri*.

Acinetobacter calcoaceticus. The gram-negative *Acinetobacter calcoaceticus* has been shown to share several of its transformation characteristics with gram-positive streptococci and *B. subtilis*. In particular, DNA competition studies of *Acinetobacter calcoaceticus* BD4 showed that DNA binding and uptake are not specific for DNA of the same species (198, 271). Competition experiments also indicated that plasmid DNA competed with homologous chromosomal DNA for binding and uptake. Apparently, plasmid DNA is taken up via the same route as is homologous chromosomal DNA. Heat-denatured DNA did not transform, suggesting that single-stranded DNA is not adsorbed or taken up (271). DNA-binding assays were performed by measuring the frequency of transformants obtained when competent cells were incubated with homologous chromosomal DNA for various periods. Nonadsorbed DNA was removed by centrifugation, and DNA uptake was determined by DNase I treatment. The results suggested a rapid and wash-resistant association of DNA with the surface of competent *Acinetobacter calcoaceticus* cells. Within 5 min, 60 to 65% of the DNA was adsorbed and was found attached to the cells after 15 min (198). *Acinetobacter calcoaceticus* requires divalent (Ca^{2+}, Mn^{2+}, or Mg^{2+}) but not monovalent cations for transformation (198). Accordingly, EDTA inhibits transformation (271). Binding of DNA to cells proceeded in the absence of any added cations (in deionized water), which led to the conclusion that only the uptake step requires divalent cations (198). Direct evidence for entry of the donor DNA as single-stranded material has been obtained in uptake studies with a *recA* mutant of *Acinetobacter calcoaceticus* (271). Similar to *S. pneumoniae*, transformation of *Acinetobacter calcoaceticus* by plasmid monomers approximated a two-hit dependence on DNA concentration (42), whereas transformation by DNA of an unfractionated plasmid preparation (293), presumably containing oligomeric forms, and by DNA of a pUC18-derived

plasmid with cloned *Acinetobacter calcoaceticus* chromosomal DNA (271) showed a one-hit dependency. Analogous to what was found with *S. pneumoniae*, transformation of *Acinetobacter calcoaceticus* by linearized plasmid DNA was very inefficient but was strongly increased when a mixture of plasmid molecules cleaved with different restriction enzymes was used (271).

Cyanobacteria. Several unicellular cyanobacteria of the genus *Synechococcus* have been studied in detail with respect to the physiology of natural transformation. Studies on DNA competition and DNA concentration dependence of transformation suggested that *Agmenellum quadruplicatum* PR6 (now classified as *Synechococcus* strain PCC 7002) and *Anacystis nidulans* R2 (now classified as *Synechococcus* strain PCC 6301) take up DNA of any source (43, 88, 103). Plasmid oligomers were about 1,000 times more active in transformation of *Agmenellum quadruplicatum* than were plasmid monomers (35). From this it seems that similar plasmid reconstitution mechanisms are active in the gram-negative cyanobacterial species, in *Acinetobacter calcoaceticus*, and in the gram-positive streptococci and *B. subtilis*. Further analyses will show whether there is an exposed cell surface-associated nuclease activity which, analogous to that of transformable gram-positive species, releases acid-soluble material from donor DNA in an amount equivalent to that taken up as single-stranded material. Kolowsky et al. (172) determined the efficiency of integration of heterologous DNA flanking or interrupting cloned homologous DNA during transformations of *Anacystis nidulans*. They found a twofold decrease of the transformation efficiency with every 2- to 3-kb increase of the size of the heterology, independent of its location and mechanism of integration (replacement, Campbell-like). This and the fact that, similar to marker rescue in *B. subtilis* (55), integration of a foreign DNA sequence in the *Anacystis nidulans* chromosome is achieved only when cleavage occurs within the homologous segment of the transforming DNA molecule (391), prompted Kolowsky et al. (172) to suggest the presence of nucleolytic processing of donor DNA during uptake. Daniell and McFadden (62) detected 50% of the amount of pBR322 DNA bound and/or taken up by *Anacystis nidulans* as acid-soluble material, although it did not become evident whether the nuclease activity was involved in uptake. Transformation-deficient (*com*) mutants such as available in *Acinetobacter calcoaceticus* (272) would be helpful for the identification and characterization of components of the uptake machinery.

Campylobacter coli. DNA uptake and DNA competition experiments show that binding and uptake of DNA during natural transformation of *Campylobacter coli* (gram negative) are specific for homologous DNA (386). Further analysis of the process will show whether this organism recognizes an uptake sequence on DNA as *Haemophilus* and *Neisseria* spp. do.

FREE DNA IN THE ENVIRONMENT

In the environmental locations examined so far (seawater, freshwater, and sediments), free DNA has been detected. The combined data from various colorimetric detection methods and gel electrophoresis indicate that extracellular high-molecular-weight DNA is present in considerable quantities in the environment (see the section on high-molecular-weight DNA in the environment, below). The DNA seems to be mostly of microbial origin. The following sections include a compilation of findings concerned with the physiology and dynamics of the release of high-molecular-weight DNA from cells cultured in standard laboratory media and present in the natural milieu.

Release of DNA from Cultured Cells

Excretion versus autolytic release in *B. subtilis*. The possibility of a gene transfer mediated by excreted DNA in the terrestrial environment was suggested in the early 1960s because of the observation that chromosomal traits were exchanged in a DNase-sensitive process during "matings" of auxotrophic mutants of naturally transformable *B. subtilis* (359). DNA was found to be spontaneously released from the cells into the culture liquid (197, 323) or to be associated with the cell wall (26, 323). Enhanced levels of extracellular transforming activity were detected during spore germination (26), during competence development (61, 197, 323), and in the death phase (197). The mechanism by which DNA is released from *B. subtilis* includes cell lysis as well as some unknown way of externalization without interfering with cell integrity.

During germination of spores, genetic markers in extracellular DNA appear in the sequential order of chromosomal replication (26), indicating that an equivalent of a complete chromosome is released. This correlation between DNA excretion and DNA replication has led the authors to assume that the point of exit of DNA may be the membrane attachment site of the chromosome where replication starts.

A precise coincidence of the release of DNA with the development of competence was observed in other studies (61, 197, 323). High transforming activity was found to be associated with the exterior of the cells (323) and present in the culture liquid (61, 197). RNA and proteins were released along with transforming DNA, although the viability of cells did not decrease (323). Sinha and Iyer (323) isolated a mutant capable of taking up DNA but unable to release DNA and other cellular components. Thus, the release of DNA during the state of competence seems to be genetically determined. Results from two experimental approaches indicated that the competence-specific appearance of transforming DNA cannot be merely the result of lysis of part of the cell population: (i) the level of typical intracellular enzyme activities was low or undetectable in the culture fluid and did not increase at the time when the transforming activity in the fluid increased (197, 354); and (ii) a mutant has been isolated which was transformable by phenol-extracted DNA or by DNA from lysed cells but not by DNA released from competent cells (354). This finding suggests association of some unknown substance with DNA in the process of externalization. In contrast to germinating spores, extracellular DNA in cultures of competent cells does not appear in a sequential order but is enriched for specific markers (61). Whether the release is active (excretion) or passive is not known, but it is possibly associated with a decrease in the integrity of the cell wall (61) as a result of enhanced levels of autolytic activity during competence (397). Export of DNA via the uptake sites of competent cells has been excluded (323). The release of DNA is not specific for chromosomal DNA. In cultures of *B. subtilis* strains with plasmids, a peak of transforming plasmid DNA appeared during development of competence (197). In the precompetence phase, a pulse of extracellular DNase released from the cells preceded the release of DNA (197). The DNase could have the effect of clearing the habitat from heterologous DNA which would otherwise compete with transforming DNA because of the nonspecific DNA uptake of *B. subtilis* (160). It is tempting to suggest that a strategy exists for highly coordinated transformational gene transfer in *B. subtilis*.

As growth of a *B. subtilis* culture in broth progressed to the stationary phase, cell lysis and concomitant DNA release occurred with a maximum in the death phase (197). The concentrations of DNA reached 3 $\mu g\ ml^{-1}$, and the highest

transforming activities of chromosomal and plasmid markers during the growth cycle were obtained. Cellular lysis is known to be preceded by the destabilization of the murein sacculus catalyzed by a cell wall-associated autolysin activity (397). Recent biochemical and molecular analyses revealed that the major autolysin *N*-acetylmuramoyl-L-alanine amidase (the product of the *cwlB* gene) is stimulated threefold when combined in a 1:1 molar ratio with the modifier protein, which is the product of the *cwbA* gene (175). Lysis is enhanced by high concentrations (150 mM) of various monovalent cations (e.g., Na^+ and K^+) and counteracted by the simultaneous presence of divalent cations (e.g., Mg^{2+} and Ca^{2+}) (356). A possible mode of action of monovalent cations may be in affecting the interaction between CwlB and CwbA (356). Phosphate also induces lysis and is counteracted by glucose plus ammonium (356, 357). The physiological basis of these findings is not clear. However, a coupling of lysis with the chemiosmotic potential of the membrane has been proposed (151). In general, limitation of the C and N source (357), as well as low temperature (358), causes cells to lyse. Lysis seems to be triggered by an unbalanced synthesis of macromolecules, especially of peptidoglycan (357).

Spontaneous release of DNA from other bacteria. Essentially all bacteria examined so far release DNA during growth in standard media. Extracellular DNA was isolated from cultures of *Brucella* spp., *Flavobacterium* spp., and various *Alcaligenes*, *Micrococcus*, and *Pseudomonas* species and strains (40, 41, 360). DNA may be associated with the cellular slime layer, thereby stabilizing its structure (40). The amounts of DNA in the culture slime can exceed 40% of dry material (40). Apparently, slime-associated DNA in *Neisseria meningitidis* cultures has transforming activity (41). An extracellular DNA-RNA complex transformed the cyanobacterium *Anacystis nidulans* at a high frequency (up to 1.2×10^{-3} recombinant per viable cell [134]). High transforming activity of chromosomal DNA, presumably released by cellular lysis, was also present in the supernatant of a 3-month-old culture of another cyanobacterium, *Synechococcus* strain OL50 (206), and in a post-stationary-phase culture of *Acinetobacter calcoaceticus* (197). Reminiscent of the finding in *B. subtilis* is the appearance of transforming DNA in the cell-free filtrate of competence-developing *S. pneumoniae* cultures. The coincidence of DNA release with competence was considered indicative of a probable natural gene transfer process in this bacterium (261). Lysis was shown to depend on Ca^{2+}. Interestingly, addition of DNA prevented cellular lysis. Trombe et al. (372) proposed that feeding on nucleotides of DNA degraded during uptake might restore balanced growth conditions of otherwise nucleotide-depleted competent cells. Extracellular DNA at extraordinary high concentrations (up to 7.8 mg ml^{-1}; molecular weight, 9.6×10^6) renders cultures of *P. aeruginosa* KYU-1 and other pseudomonads viscous (124, 377). DNA is released in the death phase, most probably through cellular lysis which is regulated by the type and concentration of C and N sources (377). Autolysis of *Azotobacter vinelandii* was dependent on temperature, pH, and the competence state of the culture (262). From these results it can be assumed that bacteria in their natural habitats release abundant quantities of high-molecular-weight DNA.

Besides excretion and lysis, other mechanisms of DNA externalization seem to exist. The formation of membrane-derived vesicles (blebs) containing chromosomal and plasmid DNA has been observed in 14 gram-negative species including *Agrobacterium tumefaciens*, *Borrelia burgdorferi*, *E. coli*, *P. aeruginosa*, and *Yersinia pestis* (75). Moreover, R-plasmids were internalized by naturally competent *N. gonorrhoeae* during incubation of cells with blebs isolated from the supernatant of the donor culture. Dorward et al. (76) assumed that a similar intercellular exchange of genes may also take place in the natural habitat of this pathogen.

High-Molecular-Weight DNA in Soil, Sediment, and Water

Methods of extraction. Extraction methods to determine the DNA content in the environment have been developed and applied to samples taken from various aquatic and terrestrial locations. Most of these DNA extraction methods obtain total DNA from the microorganisms present in the sample. Often the objective is the description and analysis of the microbial community in the ecosystem. The genera and species are identified, and their abundance is determined by quantitation of their rRNA genes and genes specific for metabolic and other characteristics of the community (e.g., nitrogen fixation, *nif*; mercury resistance, *mer*; or degradative enzymes, such as *xyl*). Total-DNA extraction is also applied to the tracking of genetically engineered microorganisms introduced into the environment or microcosms or of pathogenic microorganisms in clinical samples. Furthermore, preparations of so-called environmental DNA have been used for the amplification and cloning of genes (e.g., rRNA genes). Environmental samples are treated such that cells lyse in the sample (freeze-thaw cycles, lysozyme treatment), allowing total-DNA extraction (applying sodium dodecyl sulfate solution at 70°C), which yields intracellular and extracellular nucleic acids (direct extraction). The alternative procedure involves techniques for the separation of cells from the water or soil sample prior to DNA extraction (cell extraction). This method aims at the isolation of only intracellular nucleic acids. For details of the extraction procedures, the reader is referred to a review (370) and the recent literature (36, 95, 132, 135, 139, 285, 300, 314, 328, 336, 337, 367, 373, 374, 388, 396).

The larger amounts of DNA recovered from soil by the direct procedure than by the cell extraction procedure may indicate the presence of extracellular DNA (337). Also, comparative sequence analyses of 16S rRNA genes amplified from mixed populations of freshwater magnetotactic bacteria did not match the morphotypes observed by fluorescent whole-cell hybridization, which was taken as an indication of the presence of "naked" nucleic acids in the sample originating from species no longer present in the habitat (335). The differentiation between intracellular and extracellular DNA in an environmental sample is not a trivial matter of experimentation. This is particularly true for the isolation of free DNA from soil and sedimentary environments, since treatments and procedures aiming at the efficient extraction of nucleic acids adsorbed to organic particulates and mineral components may disrupt cells. There have been no convincingly successful attempts to specifically identify and prepare indigenous extracellular DNA from soils. The only report concerned with free DNA in a freshwater sediment is that of Ogram et al. (256). The protocol involved several washings of the wet sediment sample (100 g) with 0.12 M sodium phosphate (pH 8.0). DNA in the combined extraction volumes was precipitated with ethanol and further purified by hydroxylapatite chromatography. By this procedure, as much as 1 μg of purified high-molecular-weight extracellular DNA per g of sediment was obtained (Table 2). [^{32}P]DNA added to sediment samples was recovered with a yield of 99.9%. Similar recoveries (>90%) were obtained during alkaline extractions of [^{3}H]thymidine-labeled plasmid DNA adsorbed on sand in a microcosm (301). Soils give somewhat lower yields of introduced [^{3}H]thymidine-labeled DNA (48 to 97%), especially when the soil sample contains

TABLE 2. Quantities of extracellular DNA in the aquatic environment

Sampling location	DNA concn (μg/liter)	Molecular size (kbp)	Reference(s)
Marine water			
Estuarine	9.4–11.6	ND[a]	279, 280
	6.0–44.0	ND	68
	10.0–19.0	0.15–35.2	68
Coastal	5.0–15.0	ND	67
	2.0–7.0	ND	376
Offshore	0.2–10.5	ND	67, 279
	0.2–1.9	0.24–14.3	68
	17.1	ND	376
Freshwater			
Water column			
Oligotrophic	0.5–3.2	ND	67, 279
Eutrophic	1.1–25.6	ND	67, 279
Swamp	7.8	ND	67
Freshwater sediment	1.0[b]	1.0–23.0	256

[a] ND, not determined.
[b] Measured in micrograms per gram.

large amounts of clay (300). Whether the alkaline extraction procedure or any other method removes only extracellular DNA from a sediment or soil sample or whether there is contamination by intracellular DNA as a result of cell lysis during handling and extraction has not been critically tested. There is a strong need for further experimentation to provide the methods necessary to obtain data on extracellular DNA concentrations in soils and sediments.

The determination of free DNA in the water environment has been addressed by several studies. DNA extraction from water samples is easier than extraction from soil. Water samples of usually 0.1 to 1 liter are filtered through 0.2-μm-pore-size filters to retain microbial cells and other particulate material, and dissolved DNA in the filtrate is concentrated by ethanol precipitation (67). Measurement of DNA concentration by using Hoechst H33258 (bisbenzimidazole) seemed to be less strongly affected by substances present in natural water samples than did measurement by using mithramycin (65). Because of the high sensitivity of the method, DNA double-strand-specific fluorescent dyes are preferable for the determination of DNA concentrations. To correct for fluorescence not caused by DNA or caused by packaged phage DNA, samples treated with DNase were measured in parallel. As an example, DeFlaun et al. (68) found up to 76% of fluorescence remaining after DNase treatment of estuarine and oceanic water concentrates. Introduction of internal standards of DNA are useful to estimate recovery of environmental dissolved DNA. Depending on the sampling site, 72 to 93% of the added DNA was recovered from marine water by a filtration procedure (68). Further purification by chromatography, polyvinylpolypyrrolidone treatment (removal of humic substances), and CsCl buoyant density centrifugation gave preparations of sufficient purity for the determination of the molecular weight of the extracted DNA and for the detection of specific genes by hybridization (68, 274, 277, 279).

The question whether the DNA isolated from filtered water consisted exclusively of extracellular DNA was addressed by testing early steps during the preparation of DNA for cell lysis (67). Vacuum pressures of 25 to 150 mm Hg during filtration did not increase the DNA concentrations. Also, an intracellular compound of phytoplankton, chlorophyll *a*, was not detectable, indicating that DNA originating from phototrophic cells lysed during filtration was not contributing to fluorescence. Filtration seems to be a reliable procedure for the isolation of extracellular DNA in water. However, small quantities of DNA, which are not detectable by fluorescence, from few lysing cells during sample treatment may contaminate the extracellular dissolved-DNA preparation. This makes the interpretation of data difficult when applying highly sensitive techniques (such as DNA hybridization and PCR) for the detection of genes in extracellular DNA (274). Altogether, the procedures used indicate that considerable quantities of high-molecular-weight extracellular DNA are present in various microbial habitats (Table 2).

Origin of extracellular environmental DNA. Holm-Hansen (140) concluded from his studies on determination of biomass in ocean waters that most of the DNA found was not associated with living cells but, rather, was in detrital material of less than 2 μm in diameter and in the dissolved state. DNA equivalent to 35% (at nearshore sampling sites) and even 63% (at offshore locations) of the amount of DNA in the particulate (i.e., filter-retained) material was found dissolved in water. In statistical analyses, data for dissolved DNA were best correlated with bacterial direct counts (66). A more direct approach was chosen by Paul et al. (280). They incubated marine water samples with [^{3}H]thymidine and monitored the production of dissolved macromolecules. In estuarine and open-ocean (oligotrophic) water, >56% and 78.5%, respectively, of the dissolved trichloroacetic acid-precipitable radioactively labeled material was estimated to be DNA. DNA was produced at rates of 0.9 (oligotrophic water) and between 2.9 and 18.3 (eutrophic water) ng liter^{-1} h^{-1}. The amount of dissolved DNA produced daily in oligotrophic and eutrophic water made up 3.1% and 0.7 to 4.4%, respectively, of the extracellular DNA pool (0.7 and approximately 10 μg liter^{-1}, respectively). The highest correlation was obtained between DNA in the particulate fraction and DNA dissolved in water, suggesting that dissolved DNA originated from the filter-retained particles (277). Since bacterioplankton constitutes 71 to 91% of the particulate DNA pool in oceanic water, it is believed to be an important source of extracellular DNA (273). In coastal and estuarine environments, phytoplankton, which can contain up to 60% of total particulate DNA, may significantly contribute to dissolved DNA (273, 277).

Experiments involving a microcosm of an aquatic environment indicated that DNA is released similarly by normal and genetically engineered microorganisms. Paul and David (275) studied extracellular DNA production by [^{3}H]thymidine- or [^{3}H]adenine-labeled bacteria added to raw or sterilized marine water. Absolute DNA concentrations were not determined; rather, rates of DNA production were compared among *E. coli* strains harboring either a natural plasmid (R388) or a Tn*1721* insertion derivative thereof. A similar increase of trichloroacetic acid-precipitable radiolabel in the dissolved fraction of filtered seawater over 4 h was observed with both strains, irrespective of whether natural or autoclaved water was used. This indicates that the genotype (wild type or genetically altered) of the strains and the ambient microbial population had no measurable effect on the release of DNA from the introduced bacteria. Extracellular DNA also appeared in microcosms containing *Pseudomonas cepacia* or *Bradyrhizobium japonicum* with chromosomally inserted *nptII* conferring resistance against kanamycin (275). Highest rates of dissolved-DNA production by *P. cepacia* were obtained at low salinity and at pH 7.0 and 37°C. The data may be interpreted to indicate that DNA release in the aquatic environment may be a normal physiological performance of cells in bacterial communities.

Processes which promote DNA release from microbial cells

in the environment have been found to depend on both abiotic (e.g., ionic strength, pH, temperature [275]) and biotic factors. Recent observations have stressed the role of viruses in the ecology of microorganisms living in aquatic environments. Between 3×10^3 and 1.5×10^8 virus particles ml^{-1} were detected by electron microscopy (for a review, see reference 27), some of them found attached to and inside of bacterial cells (21, 289). The concentration of viruses was subject to seasonal variation, indicating a dynamic virus production (27). It seems that viral attack is an important factor in bacterial mortality and release of high-molecular-weight cytoplasmic components, including DNA (28, 31, 289). This assumption is substantiated by the finding that various isolates of phages lyse many bacterial strains other than the originally identified host (for a review, see reference 27). Other studies suggest that the grazing activity of protozoa contributes significantly to bacterial mortality (130, 217, 319, 376, 390). Turk et al. (376) observed a strong covariance between the number of nanoflagellates and the concentration of free DNA during incubation of northern Adriatic Sea water over several hours. When the predators were excluded by filtration in continuous-culture experiments, 3.5 μg of DNA $liter^{-1}$ appeared in water, the DNA release perhaps being caused by phage-induced cell lysis. A sixfold increase in dissolved-DNA concentration was measured when nanoflagellates were added. The results were interpreted to indicate that the majority of ingested bacterial DNA was ejected during consumption of bacteria by the nanoflagellates. Viral infection and predation by protozoa are probably the main cause of the presence of extracellular DNA in the aquatic environment. It is conceivable that similar biotic parameters may also influence the release of DNA in soil and sediment. There are several reports (reviewed in references 87 and 350) indicating a significant role of protozoa in bacterial mortality in soil.

Bacteria in natural habitats are mostly starved, and various species have been reported to enter the viable-but-nonculturable status (for reviews, see references 161, 227, and 304). Viable-but-nonculturable cells preserve their genetic information, as documented by the finding that a recombinant plasmid remained stable in nonculturable *E. coli* cells during 28 days in an artificial seawater microcosm (36). This implies that viable-but-nonculturable cells can be the source of intact extracellular DNA when, after long periods, their integrity may be lost.

FATE OF EXTRACELLULAR DNA IN THE ENVIRONMENT

Generally, nucleic acids are hydrolyzed at substantial initial rates when introduced into wastewater (90, 284), seawater (18, 66, 210, 211, 280, 376), freshwater (279), sediments (210, 211, 252), and soils (49, 111, 113, 300, 303). DNA-degrading microorganisms can be readily isolated from environmental samples. Up to 10^5 DNA-hydrolyzing bacteria ml of $seawater^{-1}$ have been found (211). Sometimes DNase-producing microorganisms in the aquatic and soil environment may make up >90% of the heterotrophic bacterial CFU (113, 211). On the basis of their data, Paul et al. (280) suggested that DNA hydrolysis was effected by the concerted action of dissolved and cell-associated nucleases. In some marine environments, most of the DNase activity seemed to be associated with the particulate fraction (210, 280).

Although DNases are ubiquitous in the environment, extracellular DNA has been found at all sites analyzed so far (Table 2). The presence of extracellular DNA in soils is assumed (see the section on high-molecular-weight DNA in soil, sediment, and water, above). A method for its extraction without destruction of cells is not yet available. One reason for the presence of free DNA in the environment may be the continuous production of DNA by organisms (see preceding section). Further, several factors that contribute to the persistence of extracellular DNA have been identified.

Protection of Extracellular DNA

Particulate constituents of soils and sediments, such as quartz, feldspar, and clay minerals, and particulate material suspended in natural water possess sorptive capacities for organic and inorganic material. For instance, proteins form tight complexes with edges and interlayer surfaces of clay minerals homoionic for various mono- and multivalent cations (cf. references 47 and 307). Binding even proceeds when the net charge of the clay minerals and of the proteins is negative (for a review, see reference 350). Once proteins are adsorbed on clay particles, their utilization by soil microorganisms appears to be reduced or abolished (350). Similarly, hydrolysis of amino acids (64) or carbohydrates (209) by soil microorganisms was shown to be retarded in the presence of clay minerals. From studies on the content of purines and pyrimidines and its fluctuations in soil, Anderson (7, 8) obtained data suggesting that a fraction of the soil DNA, which seems to be mainly of microbial origin, is not associated with bacterial cells, i.e., that the DNA may exist in an extracellular state. Hence, some portion of DNA must resist the rapid hydrolysis by soil-borne nucleases (113). Because DNA forms complexes with several clay minerals (106, 112), this was thought to be a cause of the protection of DNA against DNases and the persistence of DNA in soil (113). DNA also binds to other minerals such as quartz, feldspar, and heavy minerals (199). Humic acids are other candidates for an association with DNA. Up to 10% of total organic phosphate in soil comes from DNA bound to humic acids (12). Furthermore, when isolated from soil, DNA is frequently complexed with humic substances (373–375). Historically, investigations on the adsorption and decay of organic molecules, including DNA, on the surface of soil particulates were aimed at the understanding of nutrient cycling in soil. Along with inositol phosphate, phospholipids, and sugar phosphates, nucleic acids form an important part of the organic phosphorus pool in soils (7, 12, 327). Since the beginning of the debate on the safety of genetically engineered organisms and the growing interest in molecular and genetic aspects of microbial ecology, extracellular DNA in soil has been regarded as a source of genetic information rather than solely a source of phosphorus, nitrogen, and carbon.

DNA adsorption on soil and sediment minerals. Soil is one of the most complex microbial habitats, differing from others by its dominance of solid surfaces and spatial as well as temporal fluctuations of aqueous and gaseous phases (for a review, see reference 350). Soil consists of solid particles, which are associations of minerals (e.g., quartz, feldspar, clays) and organic polymeric substances (humus). Between the solid particles are dispersed water, inorganic ions, low-molecular-weight organic molecules, and gases (228). Because of this complexity, a promising strategy is to study DNA adsorption phenomena at the solid/liquid interface with selected components of soils and sediments in order to analyze biotic and abiotic factors which may influence the binding and degradation of the DNA. Such studies have been performed in microcosms. These are usually samples of environmental material (soil, sediment, water) taken to the laboratory and used as enclosed systems for microbiological, ecological, and other investigations (9). Microcosms can also contain purified components of natural material or model materials (e.g., artificial

TABLE 3. Adsorption of linear duplex DNA to soil minerals[a]

Mineral and cations	Concn (M)		pH	Time (min) for >50% maximum adsorption	Capacity (μg of DNA/g of material)
	Total	Half-maximum adsorption			
Quartz sand[b]					
Na^+, K^+, NH_4^+	0.05–4	1	5–9	1–15	19
Mg^{2+}, Ca^{2+}	0.001–0.2	0.015	5–9	1–15	64
Clay minerals[c]					
Na^+	0.1–5	0.5–1.3	5.2–8.9	1–5	≤13,700
Mg^{2+}, Ca^{2+}	0.0005–0.2	0.005–0.015	5.2–8.9	1–5	≤45,000

[a] Data are taken from references 166, 199, 204, 270, and 301.
[b] Composition: feldspar, 12% (wt/wt); quartz, 86.1%; heavy minerals, 1.9% (199).
[c] Natural Na-, Ca-bentonite; kaolinite; montmorillonite (made homoionic for Ca^{2+} or Na^+).

seawater, fractions of minerals). Microcosm studies have revealed a variety of parameters which determine the rate and extent of adsorption of dissolved DNA to minerals. These parameters include the type of the mineral, the valence and concentration of cations, the pH of the bulk phase, and the time needed for binding of certain amounts of the DNA to the substratum (Table 3) (see below). Temperature (199), the conformation (supercoiled, relaxed circular, linear [301, 302]), and the molecular size of the DNA molecules (for plasmids of 2.4 to 17.3 kb [42, 270, 302]) have a minor effect, if any, on the binding of DNA to minerals.

The electrostatic repulsion has to be reduced to allow the adsorption of an anionic polymer (DNA) to negatively charged surfaces of quartz or clay minerals. According to the theory of polymer adsorption (for a review, see reference 136), the equilibrium of adsorbed and free polymer molecules depends on the equilibrium of electrostatic repulsion and nonionic adsorption energy (van der Waals forces). Repulsion forces may be decreased by an increase in the concentration of counterions (cations) or a decrease in the pH. Accordingly, adsorption of DNA to various purified minerals has been found to increase with increasing monovalent (Na^+, K^+, NH_4^+) and divalent (Mg^{2+}, Ca^{2+}) cation concentrations (112, 199, 204, 270, 301) (Table 3). The characteristics of the dependence of DNA absorption on monovalent cation concentration vary among the minerals studied. For instance, an increase in the amount of linear duplex DNA bound to purified quartz sand (composed of quartz, feldspar, and heavy minerals [Table 3]) was observed after an increase in the NaCl concentration from 0.1 to 4 M (199). In contrast, DNA did not bind to natural sodium bentonite (75% montmorillonite) at ≤0.2 M NaCl (204, 270). At higher NaCl concentrations, a steep rise in the extent of adsorption occurred, with saturation of the clay by DNA at ≥2.5 M NaCl (204). Divalent cations are substantially more efficient mediators of adsorption of DNA to silicates than are monovalent cations. A $MgCl_2$ concentration 70 to 100 times lower than that of Na^+ permits the same extent of adsorption of DNA (199, 204, 301) (Table 3). The high binding potential of quartz, feldspar, and clays in the presence of low concentrations of divalent and presumably other multivalent cations in the bulk phase can be explained by a bridging action (112; for theory, see reference 136) exerted by the Mg^{2+} or Ca^{2+} ions complexing between the phosphate groups of DNA and the silicate anions of the minerals (192). Bridging may also account for the adsorption of DNA to Ca^{2+}-loaded montmorillonite in distilled water (166). In a microcosm consisting of sterilized material of a natural groundwater aquifer (0.7 g) and natural groundwater, there is almost quantitative adsorption (in 1.5 h at 23°C) of up to 1 μg of supercoiled or linear DNA from plasmids of different sizes (2.4 to 17.3 kb [42, 301, 302]). For a 10-kb plasmid the proportion of 1 μg of DNA adsorbed corresponds to the plasmid content in 4×10^9 cells (at 25 copies per cell). Multivalent cations (Mg^{2+}, Ca^{2+}, $Fe^{2+/3+}$, and Mn^{2+}) present in groundwater at a net concentration of 1.2 mM promoted the adsorption of DNA to groundwater aquifer material (42, 302). The capacity of aquifer material for plasmid DNA increased twofold when the concentration of Mg^{2+} in the bulk phase (groundwater) was raised by 5 mM (302). In addition to the ions in the groundwater, the aquifer material (98.5% sand, 0.9% silt, 1.5% kaolinite and illite) was covered with precipitates of magnesium, calcium, iron, manganese, aluminum, and copper, which in the form of exchangeable cations may also contribute to the binding of DNA to the "dirty" surfaces of the particles (302). Multivalent exchangeable cations were released from several dry soils, upon wetting (water saturation), at net concentrations of 0.6 to 2.1 mM (204), which is sufficient for DNA adsorption to minerals. The ubiquitous presence of multivalent cations in the environment and their superior role as mediators of DNA adsorption to surfaces is probably the reason for the binding of DNA by soils (257) and sediments (349).

DNA binding to mineral surfaces proceeds over a wide range of pH values (Table 3). Greaves and Wilson (112) observed a four- to eightfold increase in the amount of DNA adsorbed on montmorillonite homoionic to Na^+, K^+, Mg^{2+}, and Ca^{2+} when the pH of the solution was decreased from 5 to 3.5. In the range pH 5 to 9 the extent of DNA adsorption did not vary greatly. This was observed also with other clay minerals (166, 204). The enhanced binding capacity of montmorillonite for DNA at pH < 5 was explained by the protonation of bases, leading to a progressive increase in the positive charge of DNA and, because of the reduced distance to the surface, to increased coulombic attraction between the cationic groups of DNA and the negatively charged clay (112). Secondary physical forces (van der Waals forces) may also contribute to the binding of DNA at acidic pH. Whereas cationic adsorption mostly involves interaction of DNA with surfaces in the interlayer region of clay minerals, binding at pH 5 to 9 seems to be confined mostly to the edges of the clay (112). Here, surface exchangeable divalent cations may be responsible for adsorption of DNA. Ogram et al. (257) studied the effect of pH on adsorption of DNA to a sterilized silty soil (containing 10% clay consisting of montmorillonite and illite). At the soil-specific pH of 7.2, the saturation isotherm was of the Langmuir type (maximum adsorption, 15.4 μg g of $soil^{-1}$). Acidification to pH 5.8 resulted in extensive binding of DNA (up to 200 μg g of $soil^{-1}$), whereas at pH 10 DNA was not adsorbed. In the

experiments, DNA was added in standard saline citrate, which has a net concentration of 0.195 M Na^+. Citrate may have complexed multivalent cations in the soil, which could have reduced the binding of DNA. Studies with sodium-bentonite (75% montmorillonite), in the range of pH 5.2 to 8.9, showed that DNA did not bind to the clay in the presence of 0.1 M NaCl (204). This indicated strong repulsive forces. In contrast, almost 100% of the DNA was adsorbed in the presence of 5 mM $MgCl_2$. Thus, the divalent cations were much more effective than were equimolar concentrations of monovalent cations (204). It was argued that divalent cations promote association of negatively charged DNA with negatively charged surfaces by exerting a bridging effect (199). The chemical (cation-bridging) and van der Waals binding forces, which probably effected the association of linear duplex and supercoiled DNA with quartz sand at neutral to alkaline pH (199, 301), may also have promoted DNA adsorption to soils (257).

When adsorption of DNA to the minerals takes place, it is a rapid process (Table 3). The time required for half-maximum adsorption to purified sand and clays and to sediment material (groundwater aquifer) lies between $\leq$1 and 15 min at 23°C (112, 166, 199, 204, 301, 302). Once formed, complexes of DNA and clay, DNA and sand, and DNA and aquifer material are stable. About 75% of DNA initially bound on montmorillonite at pH 4.5 was present upon a shift to pH 7 (112). Khanna and Stotzky (166) determined the portion of DNA remaining on calcium-montmorillonite after eight 1-ml washes of a 1-mg sample with buffer or distilled water as 54 to 58%. In a flowthrough microcosm system, 82% of plasmid DNA initially adsorbed on 0.7 g of purified sand was present after 1 day of elution (12 ml h^{-1}) with $MgCl_2$-containing buffer and 63% was present after 9 days (301). Only 8% of DNA initially adsorbed on groundwater aquifer material was removed during 8 days of elution with artificial groundwater (302). In a model soil system, elution with solutions containing 1 M NaCl, a divalent cation-complexing agent (EDTA), or detergents (sodium dodecyl sulfate, Brij 58, Tween 80) still left 76, 41, and 35 to 41% of the DNA on the purified sand minerals, respectively (199). Apparently, the DNA quickly forms a tight association with surfaces of soil minerals.

The interactions between DNA and sand and DNA and clay minerals seem to be similar, irrespective of whether the material is "clean" or "dirty." However, clays have an approximately 700-fold-higher binding capacity than does quartz sand (Table 3). This may be explained by the higher surface area of clay minerals as a result of the micelle structure of the clay particles and the high net negative charge (for a review, see reference 350), allowing more DNA molecules to be bound via complexed multivalent cations. Also, as mentioned above, DNA may find access to the interlayer region of clay minerals at acidic pH (112). Recently it was found that in a sand-clay microcosm, 60% of the adsorbed DNA was located on bentonite clay, which made up only 0.6% (wt/wt) of the mineral material (204). The greater extent of DNA adsorption to a sandy soil, which consisted of quartz sand with 3.2% montmorillonite, than to acid-washed sand (no clay) may occur because of the high binding capacity of the clays (257). In conclusion, the high binding capacity of minerals, the ubiquitous presence of multivalent cations as binding mediators, and the stable association of DNA with minerals suggest that most extracellular DNA in soils and sediments exists immobilized.

DNA on particulate material in aqueous systems. Suspended particulate material in aquatic environments consists of aggregates of live cells, dead cellular material (detritus), and inorganic solid matter. Several lines of evidence suggest that extracellular DNA may be associated with detrital material. Holm-Hansen (140) inferred from the lack of correlation between DNA amounts and phytoplankton biomass parameters that extracellular DNA exists in detritus. For marine water sampled at certain locations, only 35% of DNA added as an internal standard to the samples was recovered dissolved after filtration (68). This suggests that DNA became adsorbed to the particulate material and thereby was immobilized. Further, large amounts of DNA were found in the cell debris in wastewater (90). Clearly, more experimentation is needed to settle the characteristics of DNA adsorption to suspended particulate material in the aquatic environment.

Resistance of adsorbed DNA against enzymatic degradation. Collectively, the data discussed in the preceding section suggest that DNA released from cells can bind rapidly and extensively on solid material. Several methods have been used to measure the effect of DNase on DNA adsorbed on pure sand, clay, or natural mineral material and to compare the results with the effect on DNA free in solution. Following the initial finding that DNA adsorbed on minerals is partially resistant to degradation by DNase I and thereby maintains its transforming activity for naturally competent cells (1, 195, 199, 301), studies on this phenomenon were performed. The simplest method is to mix a DNA solution with a suspension of clay mineral and, after removal of nonadsorbed DNA by centrifugation, to add DNase I to the resuspended clay. Paget et al. (270) used electrophoresis in an agarose gel to analyze clay-adsorbed supercoiled plasmid DNA after DNase I treatment. They found that, at a concentration of DNase which hydrolyzed free plasmid DNA completely, clay-bound DNA appeared as relaxed and linear molecules. In a quantitative approach, which included the detection of plasmid DNA by the artificial transformation of $CaCl_2$-treated *E. coli* cells, it was shown that DNase I reduced the frequency of transformation by clay-adsorbed DNA and free plasmid DNA by 100- and about 10^5-fold, respectively. Hence, there was some protection of clay-adsorbed plasmid DNA against enzymatic inactivation. In another study involving a similar clay-DNA model and natural transformation of *B. subtilis* as the detection system, a ca. 10-fold-higher DNase I concentration was required for inactivation of montmorillonite-adsorbed chromosomal DNA than of free DNA (166). Other experimental systems for the study of the degradation of adsorbed DNA consisted of glass columns filled with minerals or natural soil or sediment (200). These flowthrough systems have the advantage that solutions and media can be changed and nuclease solutions and/or suspensions of bacteria can be added without handling of the column contents, thus avoiding mechanical disturbance of the DNA-mineral association and loss of the transforming activity of adsorbed DNA as the result of shear forces (206). Also, relatively complex mixtures of minerals such as sand and clay can be prepared and stably maintained in the microcosm (204). Several studies with purified sand, components of sand (quartz, feldspar, and others), and natural material (soils and groundwater aquifer material) indicate that DNA adsorbed on these materials is considerably less susceptible to enzymatic hydrolysis than is dissolved DNA (1, 195, 199, 301, 302). By measuring acid-soluble reaction products, Romanowski et al. (301, 302) showed that DNA adsorbed on sand or natural groundwater aquifer material required DNase I concentrations 100- to 1,000-fold higher than that of DNA in solution required for degradation to the same extent. In a sand-clay microcosm, which consisted of 0.7 g of purified sand grains covered with 4 mg of sodium bentonite, only 36% of adsorbed high-molecular-weight linear duplex DNA (6.7 μg) was degraded within 1 h at 23°C by DNase I at a concentration (100 μg ml^{-1}) which resulted in complete degradation of free DNA (204). Interest-

ingly, the portion of DNA bound on sand was markedly less degraded (9% of 2.6 μg adsorbed) than the portion of DNA bound on clay (52% of 4 μg adsorbed). Aardema et al. (1) studied the degradation of free and sand-adsorbed *B. subtilis* chromosomal DNA by determining its transforming activity in naturally competent *B. subtilis* cells. A DNase I concentration of 0.2 μg ml^{-1} was sufficient for complete inactivation of free DNA within 10 min at 21°C. In contrast, transformants (0.2% of the control transformation frequency) were still obtained with sand-adsorbed DNA after treatment with DNase I at 8 μg ml^{-1}. Kinetic studies showed that the time required for total inactivation by DNase I (10 ng ml^{-1}) increased from 2 h for dissolved DNA to 6 h for adsorbed DNA (1). Other kinetic studies with transforming DNA indicated the existence of two types of DNA-sand associations (type I and II). DNA in both associations differed in its susceptibility to DNase I but was more resistant to degradation than was DNA in solution (199). The initial inactivation rates of the transforming activity of DNA by DNase I (10 ng ml^{-1}) at 23°C for type I and II DNA-sand associations were about 20 and 4.8% min^{-1}, respectively (45 to 56% min^{-1} for free DNA). The more DNase-susceptible type I DNA-sand association dominated (90%) when DNA was adsorbed in the presence of 50 mM $MgCl_2$. At $MgCl_2$ concentrations lower than 40 mM, the DNA-sand association was of the highly resistant type II association. Type I was convertable to type II by elution of the microcosm with low $MgCl_2$ concentrations or with EDTA, indicating that the concentration of Mg^{2+} determines the mode of DNA-sand association, which in turn determines the availability of the DNA for DNase I (199). Perhaps the extent of Mg^{2+} bridging influences the sensitivity of the bound DNA to DNase. Large polymers adsorb in a way that intramolecular segments (trains), which are in contact with the surface, alternate along the bound molecule with free segments (loops) protruding into the solution (136, 152, 295). Accordingly, electron-microscopic observations showed that only parts of DNA molecules were adsorbed to clay (270). Possibly the loops are the sites which are susceptible to DNase I (192). A double-strand break introduced into a loop would reduce the molecular weight of the DNA molecule, thereby decreasing or destroying its transforming activity. In support of this hypothesis was the finding that plasmid DNA adsorbed on pure sand or natural groundwater aquifer material was hardly hydrolyzed to acid-soluble products by DNase I at concentrations up to 10 μg ml^{-1} (301, 302). However, the supercoiled plasmid DNA adsorbed on sand, groundwater aquifer material, or clay minerals was found to be converted to the relaxed and linear forms by DNase I (270, 301, 302).

It should be pointed out that all studies on the protection of DNA by mineral association were carried out with bovine pancreatic DNase I. Recently, in similar studies, a bacterial extracellular DNase was used. The enzyme, having DNase and RNase activities, was the extracellular nuclease of the ubiquitous bacterium *Serratia marcescens* (249). The nuclease introduces single- and double-strand breaks into duplex DNA and has 50% of its maximum activity (at 37°C) at 4 and 50°C (2). Adsorption of DNA on groundwater aquifer material substantially increased the resistance of DNA against the *Serratia* nuclease (2).

The mechanism of protection of adsorbed DNA against nucleolytic degradation is not fully understood. It has been proposed that DNase finds only limited access to the adsorbed DNA molecules (166, 199, 204, 270, 301). Alternatively, since DNase itself adsorbs to sand and clay (166, 194), the nuclease may be inhibited or inactivated in a similar way to that observed with several other clay-adsorbed enzymes (307). The authors speculated that the reduction of enzymatic activity may be attributed to the masking of the active site upon binding of the enzyme to the surface of a mineral particle or to conformational changes, negatively affecting kinetic properties of the enzyme. In summary, the studies with model soil systems and mineral material taken from the environment suggest that DNA, upon release from cells in soils and sediments, can subsequently adsorb on the surfaces of mineral particles and thereby may gain increased resistance against nucleolytic degradation.

TABLE 4. Half-lives of DNA in various environments

Location	Half-life (h)	Reference(s)
Aquatic environment		
Wastewater	0.017–0.17[a]	284
	0.23[b]	90
Freshwater		
Oligotrophic	4.2[c]	279
Eutrophic	5.5[c]	279
Marine water		
Estuarine	3.4–5.2[c]	279, 280
	5.5[b]	66
Ocean surface		
Oligotrophic	12.8[c]	279
P limited	4.5[c]	376
Not P limited	45.0–83.0[c]	376
Marine sediment	235[d]	252
	140[e]	211
Terrestrial environment		
Soil		
Loamy sand soil	9.1[e]	300
Silty clay soil	15.1[e]	300
Clay soil	28.2[e]	300

[a] Conversion of supercoiled into relaxed-circular or linear plasmid DNA.
[b] Loss of hybridization signals of plasmid DNA in Southern transfers or dot blots.
[c] Loss of acid-precipitable material (colorimetric DNA determination or ^{32}P-labeled plasmid DNA measurement).
[d] In dead cells; degradation measured as in footnote *c*.
[e] Loss of the transforming activity of plasmid DNA.

Degradation of DNA in the Environment

The DNA-degrading capacity in microbial habitats can be estimated by measuring the loss of the integrity of substrate DNA in microcosms. For such experiments the DNA should be used at concentrations which approximate the concentration of extracellular DNA present in the environment. Kinetic data obtained from such studies give estimates of the velocity of degradation in a given natural environment. Table 4 summarizes the half-lives of free DNA determined in samples of various aquatic and terrestrial environments. When not presented in the original articles, half-lives have been calculated from the kinetic data available. One should be aware that different measures of degradation of DNA have been used in the various studies, namely (i) the decrease of acid-precipitable material, (ii) the conversion of supercoiled into relaxed and linear DNA molecules, (iii) the loss of hybridization signals, and (iv) the loss of transforming activity of the DNA. The loss of the transforming activity of a plasmid DNA molecule may be the result of a single double-strand break (52, 53), whereas the appearance of acid-soluble products indicates extensive hydrolysis to mono- and oligonucleotides.

Wastewater, freshwater, and marine water microcosms. When supercoiled plasmid pBR322 DNA (5 μg) was added to samples (0.1 ml) of wastewater from several stations of a

municipal wastewater treatment plant, the DNA was completely converted to the open-circular and linear forms within 20 min, with only low seasonal variation (284). Similarly, the physical integrity of a recombinant plasmid designed for the large-scale production of human erythropoietin in mouse cells was rapidly destroyed (half-life, 13.8 min) in the wastewater of a fermentation plant (90). The half-life of the plasmid was measured by Southern transfer hybridization. Plasmid degradation was also monitored by loss of its transforming activity for Ca^{2+}-treated *E. coli* and by PCR amplification of an erythropoietin-specific 671-bp target sequence. In another wastewater sample, the same plasmid DNA was totally degraded within 5 min. Hybridizable plasmid DNA fragments were detected in the debris fraction of wastewater, indicating a somewhat slower degradation of adsorbed DNA or enhanced binding of DNA fragments to debris material. Generally, high-molecular-weight DNA seems to be destroyed within minutes by wastewater-borne nucleases (Table 4).

In natural freshwater and marine water, DNA is degraded with half-lives of several hours (Table 4). In contrast to wastewater and sludge, which contain viable counts of up to 10^9 of metabolically active microorganisms ml^{-1} (24), bacteria in surface water are mostly starved, as indicated by a 10- to 100-fold-higher direct count than viable count (310). Turk et al. (376) determined the rates of DNA degradation in a P-limited and in an N-limited (high-phosphate pool) marine environment. In the P-limited water, DNA degradation rates were more than 10 times higher than in the N-limited water (Table 4) and resulted in an elevated level of dissolved phosphate which was used by the nano- and picoplankton. It seems that the trophic status of these environments determined the rate of DNA hydrolysis. In contrast, in an oligotrophic and a eutrophic freshwater environment, degradation rates of DNA were found to be quite similar (Table 4).

Soil and sediment microcosms. Nucleic acids are substrates for soil microorganisms. When added to soil, DNA or RNA (2 mg g of $soil^{-1}$) induced a 35-fold increase in the bacterial viable count after 3 days (113). Other experiments showed that glass slides which were covered with a thin film of RNA- or DNA-loaded montmorillonite and deposited on the surface of soil were intensively colonized by bacteria (microcolonies of mostly *Pseudomonas* spp.; actinomycetes) but also by fungi and protozoa (113). The growth of bacteria and fungi probably resulted from the products released from nucleic acids by extracellular microbial hydrolases. On the other hand, nucleic acids adsorbed on the interlayer surfaces of montmorillonite were less extensively degraded after 3 days (15%) than were those adsorbed on the edges of the clay mineral (40%). Thus, despite the production of microbial nucleases in the vicinity of the nucleic acid-montmorillonite complex, a considerable portion of DNA in soil persisted.

Romanowski et al. (300, 303) investigated the breakdown kinetics of purified plasmid DNA introduced into different nonsterile soils. The DNA was extracted from the soils after various incubation periods of up to 60 days. The results of Southern transfer hybridizations showed that the plasmids remained intact in the soils for 2 to 5 days before degradation commenced. A quantitative biological assay of plasmid DNA, i.e., transformation of Ca^{2+}-treated *E. coli*, revealed soil-specific half-lives between 9.1 and 28.2 h (Table 4). More-sensitive methods, namely quantitative PCR amplification of a plasmid gene and electroporation of *E. coli* with DNA extracted from soil, showed biphasic degradation kinetics of plasmid DNA in the soils (303). After an initial phase of 10 to 20 days with relatively high degradation rates, the rates of inactivation of transforming plasmid molecules and decrease of the number of plasmid genes leveled off. With PCR, 0.01 to 0.2% of the initially added target genes were detectable in the soils after 60 days of incubation. The transforming activity, a measure of the presence of intact circular plasmid molecules, was 0.011% of the initial transforming activity after 60 days in a loamy sand soil, 0.004% after 40 days in a clay soil, and 0.002% after 10 days in a silty clay soil. These results suggest that transformation-active DNA molecules including plasmids released from living or dead cells, although subjected to continual degradation, may persist in soil for weeks or even months. Long-term DNA persistence, exceeding periods found in soil, is suggested for sediments because of the strikingly long half-lives (Table 4). The data from microcosm studies indicate that the persistence of DNA in soil and sediment may rely at least partly on the rapid and extensive adsorption of DNA to mineral surfaces, which provide protection against nucleolytic degradation. Other studies indicate that DNA in dead cells may also be highly protected against enzymatic hydrolysis (252) (Table 4).

COMPETENCE DEVELOPMENT UNDER ENVIRONMENTAL CONDITIONS

Bacterial cells are challenged by continually changing environmental conditions in soil, sediment, and water. Physiological stress is produced by many factors, including nutrient limitation, varying pH and temperature and, for soil, fluctuations of available water (for a review, see reference 350), as well as by oxygen radicals, UV irradiation, and other cytotoxic agents (219). In addition, competition with other cells of the same or unrelated species for nutrients and the accumulation of extracellular metabolic products may have profound impacts on the physiological status of individual cells in a habitat. Under oligotrophic conditions bacteria can enter a state of reduced metabolic activity (dormancy) which allows them to survive long periods of adverse conditions. Dormant cells do not grow or multiply and may show morphological changes, including spores, cysts, or size-reduced forms (dwarfs) of vegetative nonsporulating species (for reviews, see references 161, 227, and 304). Dormant cells of *E. coli* have been found. These retain viability, even after storage for 38 years at room temperature, as a result of the production of specific proteins that protect cells against oxidative stress (86). At the onset of starvation, a regulatory response leads to the enhanced expression of particular metabolic genes. For instance, increased levels of glutamine synthetase appear in *E. coli* and *Salmonella typhimurium* as a consequence of nitrogen limitation (220). Other complex regulatory systems have been identified for an alternative phosphate transporter induced at phosphorus deprivation, increased substrate scavenging capacity during carbon limitation, and exploitation of iron present in only trace amounts (for a review, see reference 220). Generally, nutrient limitation, heat, or acidic conditions induce the expression of individual sets of genes whose products are believed to enable bacteria to adapt to extreme situations (129, 219, 248). A particular environmental signal may stimulate the simultaneous resistance to several stress factors. Nutrient starvation confers upon *E. coli* the ability to survive heat shock and oxidative damage (reviewed in reference 219). *Vibrio* sp. strain S14 additionally develops resistance to UV irradiation and $CdCl_2$ stress under carbon and multiple-nutrient starvation (253). These examples show that bacteria have evolved efficient strategies and mechanisms to cope with adverse environmental situations. In the following sections we will discuss the requirements for and the influence of stress factors on competence development in the environment.

Environmental Parameters

Experimentally, competence of cells is determined by their transformability. However, the measurement of transformation frequencies under a variety of conditions does not allow one to determine whether a particular environmental factor triggers the induction of competence development or is required for the development of competence following induction. Therefore, when the term "competence development" or simply "competence" is used in the following discussion, it includes the process of its induction.

Nutrient utilization and competence. Competence development requires that bacteria be in a metabolically active state. For this, suitable nutrients must be available.

Dubnau (79) discriminates between three modes of competence regulation in *B. subtilis*, namely growth stage-dependent, cell type-specific, and nutritional regulation. *B. subtilis* requires a specific medium in order to become highly competent. Growth in complex media essentially represses competence development, whereas a minimal medium-grown culture reaches the highest transformation frequency (10 to 25% of cells competent [79]). Although the precise reasons for the nutritional requirements are not known, there is an indication for a preference of glucose as the carbon source. Albano et al. (3) noted that glucose and glycerol were equally good growth substrates but that transformation frequencies were about 10-fold lower when cells were grown with glycerol than with glucose. Some of the *com* genes showed reduced or delayed expression during growth with glycerol. Expression of most *com* genes was even inhibited when glutamine was present in glucose minimal medium. Competence is regulated by several gene products, which act as components of a signal transduction cascade (e.g., ComA and ComP [79, 80]). Some of the proteins seem to sense the quality and level of nutrients (79, 80). In the presence of a poorly metabolized carbon source (succinate) or with the depletion of amino acids or phosphate (245), the proteins trigger the expression of other regulatory genes. For instance, amino acid deprivation is a signal triggering ComP-ComA-mediated expression of *degQ*, which controls the enhanced expression of extracellular degradative enzymes in *B. subtilis* (see reference 245 and references therein).

Azotobacter vinelandii is rather well characterized with respect to nutritional demands for competence development. High transformation frequencies (10^{-3} to 10^{-2}) can be achieved during growth in minimal medium, and, similar to *B. subtilis*, competence is hardly developed in complex medium (266). Page (263) studied the effect of several carbon and energy sources on competence development in *Azotobacter vinelandii*. Transformation levels were similarly high with various mono- and disaccharides when a nonencapsulated strain was examined. In contrast, an encapsulated strain showed an up to 200-fold carbon source-dependent variation in the transformation frequencies, possibly because of changes in capsule production. Capsule production was speculated to reduce binding of DNA to cell surface receptors (263). Accordingly, a decrease in the amount of capsule material in phosphate-rich medium was considered to be an explanation for enhanced competence (266). Several amino acids, ammonium salts, and N_2 were potent nitrogen sources for competence development in *Azotobacter vinelandii* as long as the medium was buffered at near neutrality (262). Also, cometabolism of glucose and a variety of organic acid salts (e.g., acetate, succinate, fumarate, citrate) allowed development of competence of a nitrogen-fixing culture. Thus, it seems that the quality of the carbon and energy source and nitrogen source is not a crucial factor for competence development of *Azotobacter vinelandii*.

Acinetobacter calcoaceticus develops competence in complex as well as minimal medium (271). The kind of carbon and energy source (lactic acid, glucose, ethanol, or hexadecane) did not influence competence (271).

A variety of substrates have been tested for their ability to promote competence in *P. stutzeri* (202). Valine, various organic acids, alcohols, and mono- and disaccharides, as well as a polysaccharide (starch), allowed competence development, with some variation in the efficiency of transformation. No competence developed with lactate or glucose as the sole carbon and energy source. A correlation between transformation frequency and growth rate on different carbon sources was not found. For instance, with valine as the carbon source, cells did not divide but a relatively high competence state was reached.

Nutrient limitation. In the environment, bacteria live mostly under nutrient-limited conditions. In soil and water, the concentrations and compositions of nutrients are occasionally subjected to large spatial and temporal variations. As a consequence, bacteria may experience periods of growth and starvation (161, 304). Therefore, it is of interest whether starvation can affect the competence state of cells.

Several studies report the influence of limited nutrient supply on competence development. In *Azotobacter vinelandii*, an optimal competence regime (i.e., to obtain a competent culture) was worked out with a medium that contained a suitable carbon and energy source (e.g., glucose, sucrose, or glycerol), phosphate, a source of combined nitrogen (e.g., ammonium acetate), and Mg^{2+} and Ca^{2+} but which was limited in iron, leading to premature cessation of growth because of iron starvation of the cells (268). Cells of *Azotobacter vinelandii* starved for iron and molybdenum reached an even higher competence level than did cells in medium limited for iron only (263). Nitrogen-fixing cells become competent only at iron limitation and when nitrogenase is protected from inactivation by oxygen, i.e., under anaerobic conditions (262). Molybdenum starvation effected competence of otherwise nontransformable mutants with a deficiency in the nitrogen-fixing (*nif*) capability. There is some evidence suggesting that development of competence in ammonium-starved cells is controlled by nitrogen catabolite derepression, presumably mediated via modulation of glutamine synthetase (267). In an attempt to estimate the ability of *Azotobacter vinelandii* to become competent in soil, which in aerobic zones of near neutral pH may contain abundant but insoluble iron, Page and Grant (265) examined competence development in a medium containing iron in the form of several insoluble minerals. They noted variable iron mobilization from the minerals by the production of siderophores. Kinetic examination showed that the appearance of transformants coincided with the production of siderophores, which is indicative of the onset of iron starvation. In the phase of iron mobilization by enhanced siderophore production, a plateau or decrease of competence depended on the mineral present. As a rule, minerals that served best as iron sources gave the lowest competence. Page and Grant (265) considered iron starvation and hence competence induction in *Azotobacter vinelandii* in soil as being probable, provided that a suitable carbon and energy source is available and other parameters are favorable (neutral pH, about 1 mM Ca^{2+}). It is interesting that the rhizosphere of plants, which is considered nutrient enriched, is an iron-limited habitat (260). A stimulatory effect of iron limitation on the competence of cells of the cyanobacterium *Anacystis nidulans* R2 was also observed (103).

The availability of a suitable carbon and energy source, a nitrogen source, and/or a phosphorus source may be critical in natural environments, nutrients often being enriched only at special locations such as soil mineral surfaces (350) or the rhizosphere of plants (240). The effect of carbon, phosphorus, and nitrogen limitation on growth and competence has been studied with two other soil bacteria, *B. subtilis* and *P. stutzeri* (202). Cells were incubated in the presence of transforming DNA on a minimal agar medium which contained various concentrations of either the substrate (*B. subtilis*, glucose; *P. stutzeri*, pyruvate), phosphate, or ammonium. Although *B. subtilis* did not develop competence (16), *P. stutzeri* became increasingly competent as growth was limited by the reduction of the nutrient concentration in the medium (202). At a specific low nutrient concentration just above the nongrowth threshold concentration of the C, P, or N source, competence reached a maximum of up to 25-fold higher than at full nutrient supply. Time course experiments indicated that competence of *P. stutzeri* appeared as growth ceased, as a result of the depletion of nitrogen, and continued to increase to reach a maximum with nitrogen starvation. When ammonium was substituted for nitrate, competence was high but the cells did not divide over the range of concentrations tested (0.02 to 10 mM). Apparently, NO_3^- was not used as a nitrogen source, and hence cells were starved for nitrogen, which in turn stimulated competence (207). The stimulatory effect on competence by limitation of specific nutrients was observed only when cells were starved for one nutrient. Limitation of two or three nutrients produced transformation frequencies similar to or, in most cases, lower than those in fully supplemented medium.

Calcium. The presence of Ca^{2+} in the medium has been shown to play a key role for competence development in two organisms. In *S. pneumoniae* Ca^{2+} is essential for growth, competence development, and autolysis. The last two effects of Ca^{2+} are under the control of the external competence factor (372). Competence is induced optimally at 1 mM Ca^{2+}. Genetic evidence for a Ca^{2+} transporter, mediating the Ca^{2+}-dependent development of competence, came from studies with a mutant which was nontransformable (and resistant to autolysis) in high-Ca^{2+} medium and which was resistant to a specific inhibitor of calcium transport, 2′,4′-dimethylbenzamil (371). Trombe et al. (372) considered induction of competence as a response to stress produced by high concentrations of Ca^{2+} (1 mM), which may prevail in the natural environment of *S. pneumoniae*, the body fluids (372).

In *Azotobacter vinelandii*, Ca^{2+} is required for competence development (264) and Mg^{2+} is required for transformation (269). Sr^{2+} can substitute Ca^{2+} but not Mg^{2+} in competence development (264). Distilled-water washes of calcium-limited cells released several proteins, one of which was competence specific, from the cells. Competence was rapidly recovered when washed cells were suspended in the original supernatant plus added Ca^{2+}, suggesting a Ca^{2+}-dependent reassembly of a transformation-related surface structure. It was assumed that a glycoprotein with a strongly acidic pI would mask negative charges and thereby allow binding of DNA (264). The Ca^{2+} concentration for optimal competence development lies between 0.5 and 1 mM, which is frequently that of exchangeable and free Ca^{2+} in the natural environment of *Azotobacter vinelandii*, i.e., soil, sediment, and water (for calcium concentrations in the environment, see reference 222).

pH and temperature. A variety of naturally transformable bacteria have now been characterized physiologically with emphasis on the response of competence development to pH and temperature. Normally, the examination of effects of physicochemical parameters on competence aims at the improvement of transformability for experimental purposes. These investigations may, however, also give some hints about the competence status of transformable bacteria in the environment.

When grown in medium with various initial pH values, *Azotobacter vinelandii* and *P. stutzeri* showed a narrow peak of transformability near pH 7.0 and a steep decline at increasing and decreasing pH (203, 262, 266). Although these data do not tell us whether competence development or DNA uptake processes are affected by pH, Palmen et al. (271), in their experiments with *Acinetobacter calcoaceticus*, distinguished between these two possibilities. Transformation frequencies fell more than 3 orders of magnitude when the initial pH of the culture was shifted from 6.7 to 5.4. Cells cultured at acidic pH and then transferred to fresh medium of pH 6.7 gave high transformation levels similar to those of cells grown at pH 6.7. Cells grown at neutral pH and subsequently shifted to pH 5.4 gave low transformation frequencies. It was concluded that competence developed at neutral and low pH but that the uptake of DNA or some later step of transformation was impaired at low pH.

In *S. pneumoniae*, an increase of the pH of the medium continuously moved the competence peak to earlier exponential phase and thus to lower cell densities (45). At pH 7.3 to 8.0, a second wave of competence followed the first between one and two generations later. In this organism, competence is induced at a specific cell density by accumulated extracellular competence factor (325). Chen and Morrison (45) explain the modulation of competence by culture pH by assuming that the production of the competence factor proceeds constitutively and is unaffected by pH but that the critical level of competence factor (which corresponds to a specific cell density) for competence induction is pH dependent. In other words, the competence factor is more active at alkaline than neutral pH. The strong pH dependence of the activity of the competence factor (366) supports this view.

There are only few studies in which the effect of temperature was examined with respect to that expected to prevail in natural sites. Plate transformation assays showed that *Azotobacter vinelandii* was transformed at fairly high frequencies between 26 and 37°C with a peak at 30°C (266). The transformation frequency of *P. stutzeri* had a plateau between 20 and 37°C and decreased exponentially between 20 and 12°C (203). At 12°C, the transformation level was 0.7% of that at 30°C (and more than 2 orders of magnitude above the spontaneous reversion frequency of the auxotrophic marker used). In *Vibrio* sp., transformation frequencies dropped by about 2 orders of magnitude as the temperature was raised from 33 to 37°C (94). This is remarkable since the growth rate is highest at 37°C. Substantial levels of transformation were obtained when cells were cultured between 15 and 33°C. The studies do not distinguish whether the development of competence, DNA uptake, or further DNA processing in the cytoplasm is temperature sensitive.

Response in Environmental Simulations

Competence in soil extract. Experiments under defined conditions suggest that there may be species-specific and even strain-specific responses to environmental factors with regard to competence development and transformation efficiency. Thus, to obtain data on transformation with relevance to bacterial life in a natural habitat, it is helpful to examine the properties of strains under environment-simulating conditions. The influence of the chemical milieu of soil on competence

development and transformation has been studied with *P. stutzeri* in soil extract media (202, 203). Washed noncompetent cells were incubated together with transforming DNA on nonselective agar media prepared with the aqueous extracts (passed through a 0.45-μm-pore-size filter to remove indigenous cells) of different soils. The media were used unamended and also amended with pyruvate, phosphate, or ammonium or with combinations of the nutrients. In growth studies (202, 203), the soil extracts proved to be limited in metabolizable carbon and energy sources and nitrogen sources, and two extracts were additionally limited in phosphorus sources. Competence did not develop in the soil extract media unless the missing nutrients were added. Then transformation frequencies reached the same level as in a typical laboratory minimal medium. It was concluded that in soil extract competence developed similarly to that in minimal medium and that transformation was not inhibited by soluble substances extracted from the soils. When the soil extract media were amended with defined nutrients, leaving the extracts limited for nitrogen or phosphorus sources, competence development was stimulated up to 290-fold compared with that observed in fully supplemented soil extracts (203). These findings, obtained with an experimental system simulating the chemical conditions in a water-saturated soil, suggest that competence development of *P. stutzeri* may occur in its natural habitat, particularly when fluctuating nutrient input causes periods when one nutrient is at a growth-limiting concentration (203).

Maintenance of competence. The question of how long the competent state of cells can be maintained once it is achieved is important for the overall probability that a cell will become transformed. Furthermore, the development of competence and the process of transformation may be affected differently by environmental parameters. For example, a population of cells may become competent but may not be transformable because of the presence of soil chemicals inhibiting a step of DNA internalization. This would not preclude transformation after a change of the milieu to conditions permissive for DNA uptake, e.g., after a shift from acidic to neutral pH (see the preceding section). Frischer et al. (94) noticed that, in *Vibrio* sp., the level of competence was reduced only about 10-fold during incubation at 29°C for 10 days in artificial seawater. A similar unexpected finding was made with *Acinetobacter calcoaceticus*. Transformation frequencies decreased only about 200-fold during incubation of competent cells in sterile or nonsterile natural groundwater at 7°C for 7 days (Fig. 1). The physiology and genetics of starving cells differ tremendously from those of growing cells (253; for reviews, see references 168a, 219, and 220), and it seems worthwhile to investigate whether competence develops and is maintained in starved cells. An observation possibly related to this point (197) was that competence of *Acinetobacter calcoaceticus* developed in the late stationary phase of a culture when the titer of viable cells had decreased by more than 3 orders of magnitude and when cells tended to lyse (DNA release). It is conceivable that starved surviving cells used material released from lysing cells for metabolism and competence development. In the cyanobacteria *Anacystis nidulans* R2 and *Agmenellum quadruplicatum*, exclusion from light (the source of energy) resulted in the rapid loss of competence (43, 88). These results suggest that a high energy load of the cells was necessary to maintain the competent state.

TRANSFORMATION IN THE ENVIRONMENT

For the transformation of bacteria in the environment, several requirements have to be met. One is that a competent cell must come into contact with a DNA molecule. Because of diffusional movement of cells and DNA molecules in liquid and because of the tendency of DNA and cells to adsorb onto solid particles, one might expect higher chances for transformation events in the water milieu than in the sediment or soil environment, where surfaces dominate. Another point is that the transforming activity of a DNA molecule depends on a minimum length. Furthermore, specific cations have to be available at appropriate concentrations as cofactors for the transport and processing of DNA (see the section on biology of natural genetic transformation, above). These aspects and the question of what happens to heterologous DNA once it is taken up into a cell have been considered in various studies and will be discussed below. In this section the data of microcosm experiments are analyzed with respect to the probability of transformation events occurring in the environment.

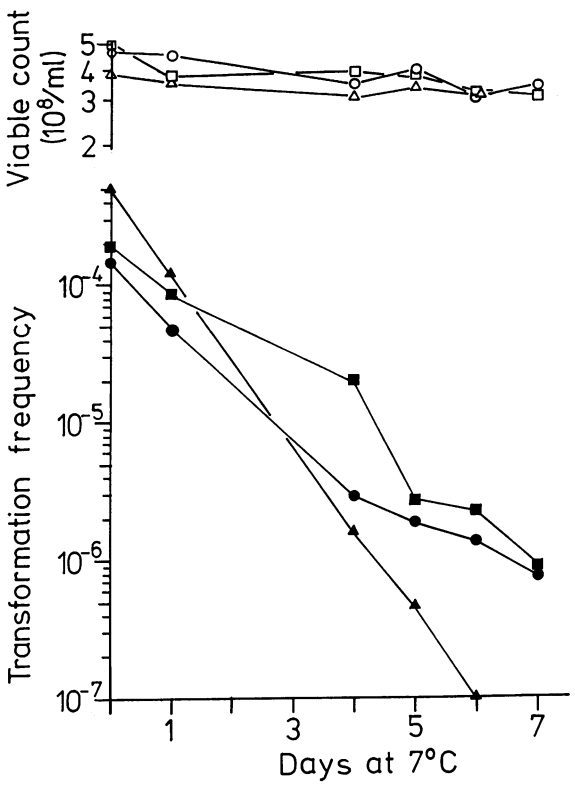

FIG. 1. Maintenance of competence in groundwater. *Acinetobacter calcoaceticus* was made competent (198), and cells were washed twice with Tris-HCl buffer (pH 7.0) and suspended in buffer containing 5 mM $CaCl_2$ (△, ▲), in pasteurized (10 min, 80°C) groundwater (○, ●), or in nonsterile groundwater (□, ■). Cells were kept at 7°C, and after the periods indicated, aliquots (0.2 ml) were withdrawn. Transformation was carried out for 30 min with 2 μg of pKT210 DNA ml^{-1} at 21°C. DNase I (100 μg ml^{-1}) was present during the period of expression (90 min) of plasmid-encoded chloramphenicol resistance. The transformation frequencies (solid symbols) and viable counts (open symbols) were determined after 1 day at 30°C.

Availability of DNA: Cell-DNA Interactions

Transforming DNA. Extracellular DNA in marine water appears to be double stranded as judged from the similar fluorescence spectra of environmental DNA and purified duplex DNA in the presence of double-strand-specific dyes (67). Other measurements with fluorescent dyes also indicate

that free DNA in the environment is mainly double stranded (68, 162, 273, 275). The double strandedness of environmental DNA would meet one important requirement for transformation (see the section on biology of natural genetic transformation, above).

The efficiency of transformation also depends on the molecular size of the DNA molecule. In *B. subtilis*, the shearing of DNA from 28.5 to 4.5 kb (mean molecular size) decreased the transformation by 100-fold, and shearing to 2 kb reduced it a further 100-fold (243). DNA of about 1 kb (mean molecular size) was inactive. In *P. stutzeri*, transformation frequencies were equally high for DNA of 10 and 60 kb but decreased more than 10-fold for DNA between 10 and 1 kb (38). Transformation frequencies of *Acinetobacter calcoaceticus* decreased in direct proportion to the mean molecular size of the DNA in the range between 40 and 10 kb (190). In instances for which data are available, free duplex DNA in water and sediment is present at molecular sizes (Table 2) suitable for transformation.

There are no reports that extracellular plasmid DNA has been searched for or found in environmental samples. As studies with cultured soil bacteria indicate (197), plasmids are released together with chromosomal DNA from cells. Such released plasmids can retain their transforming activity in soils (300, 303). If free plasmid DNA was present in microbial habitats, it would, in principle, be active in transformation (see the section on biology of natural genetic transformation, above).

Environmental DNA may not be of the high purity normally used for transformation studies. It may be complexed with material (like proteins) from lysed cells or other environmental substances. DNAs in crude lysates (156) or released from bacteria into growth medium (197) and DNAs added to groundwater or aqueous extracts of various soils (see the next section) or to marine water (278) all have high transformation activity. These data suggest that the lack of purity of extracellular DNA does not reduce its transforming activity in the environment. On the other hand, Romanowski et al. (300) observed that plasmid DNA incubated for several days in soils retained a physical stability (measured by Southern hybridization) that was not paralleled by a high transforming activity. The authors argued that unknown abiotic factors may have caused the reduced transforming activity of the plasmids.

Chemical milieu. The occurrence of transformants of a marine *Vibrio* sp. in microcosms with natural marine water (278) and of the soil bacterium *P. stutzeri* in aqueous extracts of various soils (202, 203) suggested that the chemical requirements for transformation of these two species are matched in their habitats. Except for the effect of salinity on transformation in *Vibrio* sp. (94), a detailed analysis of chemical factors influencing the transformation process of *Vibrio* sp. and *P. stutzeri* has not been performed. Lorenz et al. (198) and Romanowski et al. (302) studied transformation of *Acinetobacter calcoaceticus* and *B. subtilis*, respectively, in environmental aqueous samples. *Acinetobacter calcoaceticus* was transformed by plasmid DNA in groundwater and aqueous soil extract at frequencies similar to those under standard conditions (growth medium or buffer). The chemical analysis of the groundwater and soil extracts indicated the presence of divalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}) at concentrations which were optimal for transformation in buffer solution (198). In contrast, *B. subtilis* was hardly transformable in groundwater (302). This was attributed to suboptimal concentrations of Mg^{2+} and, particularly, of monovalent cations in groundwater. When these cations were added to groundwater, transformation was efficient (302). As mentioned in the previous section, nonenzymatic substances, inhibiting transformation, seem not to be present in the groundwater, soil liquid, and marine water column milieu.

Bacteria and solid surfaces. In the environment, cells are often associated with the surfaces of particulate material. Iriberri et al. (146) have summarized data showing that the proportion of cells attached to particles suspended in freshwater and marine water may vary between 1 and 98%. Sorbed cells were found to be metabolically more active and their concentration was less influenced by seasonal variation (cold, warm) than for free living cells. Bacterial isolates from a groundwater aquifer were found to sorb to the sandy material. About 45 and 20% of a population remained attached under continuous flow with groundwater and lake water, respectively (188). Adhesion is believed to be a survival strategy of starving cells taking advantage of scavenging nutrients enriched at surfaces (discussed in reference 169). Alternating cycles of attachment and detachment are thought to increase the chance of transport of cells to regions favorable for growth and survival (380). As an example, migration of *Azospirillum brasilense* toward grass roots was observed (13, 14), although most of the cells were sorbed on soil particulates. Bashan and Levanony (14, 15) interpreted these results to indicate that motility forces can overcome adsorption forces, thereby enabling cells to migrate in the free water films around the soil particulates. Whether surfaces generally influence bacterial metabolism in a positive way is still open to debate; if they do so, they presumably exert their influence by indirect effects of changes of the medium (e.g., removal of toxic substances by adsorption [for a review, see reference 382]). The initial contact of cells with surfaces may result in a loose reversible adhesion. The distance of sorbed cells from the surface is governed by the balance of electrostatic repulsion and van der Waals attractive forces (215). Reversible adhesion is sensitive to shear forces, so that water flow or flagellar activity may allow motile bacteria to move along surfaces (216, 242). Because of the net negative charge of many solid surfaces in the environment and of bacterial cells, adhesion to mineral surfaces such as quartz, feldspar, or clays depends on the reduction of electrostatic repulsion forces. This can be accomplished by an increase in the salt concentration (144, 216) or the hydrophobicity of the cell (340, 381). Production of polymeric material such as polysaccharide may stabilize the initial reversible adhesion of cells and may promote the formation of microcolonies and biofilms as well as the adherence of other cells to the surface (6, 56, 57, 216, 382). As outlined above (see the section on fate of extracellular DNA in the environment), DNA in sediment or soil environments probably also adsorbs rapidly on mineral and other surfaces and thereby is protected against degradation by nucleases. Is this immobilized DNA still available for uptake by competent cells? This question has been addressed by several microcosm studies with clean mineral material (quartz-enriched sand, clay) and competent cells of several bacterial species. Results will be discussed in the following section.

Transformation on solid surfaces. Experiments with flowthrough columns, filled with acid-washed and calcined sea sand, showed that competent *B. subtilis* took up adsorbed chromosomal DNA directly from the sand particles (196). The studies revealed that the transformation efficiency was up to 50 times higher at the solid/liquid interface than in liquid and up to 3,200 times higher when only the cells bound on the mineral surfaces were considered. A fraction (34%) of the sand-bound cells reversibly adhered to the surfaces of the different minerals, including quartz, feldspar, and heavy minerals. About 10 times more cells were retained in the column with DNA-

loaded sand than in columns with plain sand (1% of cells sorbed), indicating that cell adhesion was directed to sites where DNA was adsorbed. The retarded appearance of transformants in the effluent indicated that, following DNA uptake, the cells detached. Lorenz et al. (196) suggested that a tight contact occurred between sorbed cells and adsorbed DNA since DNA uptake was not affected by a dose of DNase I (1 μg ml^{-1}) which otherwise reduced transformation in liquid to 10%. The altered characteristics of transformation in the solid/liquid interface seem to be restricted to *B. subtilis*, because neither *P. stutzeri* nor *Acinetobacter calcoaceticus*, two gram-negative soil bacteria, reveal the striking enhancement of transformation on solid surfaces (42, 201). Nevertheless, both organisms were shown to be transformed on sand surfaces (42, 201). The proportion of transformants resulting from uptake of DNA desorbed from the mineral grains during incubation with cell-free filtrates of the competent cultures was less than 10% of the total *P. stutzeri* transformants (201) and about 20% (chromosomal DNA) or 8% (plasmid DNA) of total *Acinetobacter calcoaceticus* transformants (42). This clearly demonstrated that competent cells of the three soil bacteria studied are capable of internalizing DNA directly from the mineral surfaces. The efficiency of transformation by adsorbed chromosomal DNA was as high as by free DNA in *Acinetobacter calcoaceticus* and *P. stutzeri*. However, transformation of *Acinetobacter calcoaceticus* by adsorbed plasmid DNA was much less efficient than by free plasmid molecules (42). The transformation changed from a two-hit DNA concentration dependence in liquid to a three-hit process in sand columns. The possible reasons for the inefficiency of plasmid transformation on surfaces are discussed below (see the section on transformation in situ).

Clay minerals are important constituents of soils and sediments and are characterized by their high sorptive capacities. Khanna and Stotzky (166) noted that competent *B. subtilis* was transformed at a frequency almost 2 orders of magnitude lower with clay-bound chromosomal DNA than with free DNA. After removal of nonbound DNA by extensive washing with buffer or distilled water, some 20% of the initially added DNA was adsorbed on the outer surfaces of the clay minerals. When such a DNA-clay suspension was mixed with competent cells, low frequencies of transformants were obtained. Unfortunately, no efforts were made to determine whether DNA was desorbed from the clay during incubation with the competent culture. Also, desorbed DNA could still have transformed cells later on the plate, because no DNase was added to destroy any DNA not taken up by the cells at the time of plating. Other experiments have used a microcosm which consisted of flowthrough columns filled with sand and bentonite clay (204). About 60% of the applied chromosomal DNA was adsorbed on the small amount of clay (0.6%, wt/wt), demonstrating the high binding capacity of clay for DNA despite the presence of other sorptive minerals (quartz, feldspar, heavy minerals [199]). In this soil model, transformation frequencies of *B. subtilis* were similar to those in standard liquid transformations. DNA, bound on the minerals, was not removed when the clay-sand matrix was incubated with the cell-free filtrate of the competent culture, suggesting that cells took up DNA directly from the clay and sand particles. Upon separation of the DNA-loaded microcosm material into a clay and a clay-plus-sand fraction and incubation of the fractions with competent *B. subtilis*, almost 60% of the overall transformants were found to arise from clay-bound DNA. The data imply that transformation by linear duplex DNA in particle-dominated environments may take place at the solid/liquid interface of typical sediment and soil particles. The effect on transformation of the ubiquitous, highly polymerized, strongly DNA-sorbing humic substances has not been investigated so far.

Transformation in the course of cell-cell contact. Outgrowing spores of *B. subtilis* release chromosome equivalents concomitant with replication. DNA appeared in a sequential order of markers, starting with those near the replication origin (26, 259). This DNA sedimented with the cells, indicating association with the cell walls. In an attempt to study the availability of this DNA for transformation, germinating spores were coincubated with separately prepared competent cells. It turned out that the spore-associated DNA was resistant to high doses of DNase I (259), in contrast to DNA extracted from cells. When the outgrowing spores were treated with 2 mg of DNase I ml^{-1} prior to cocultivation with the competent recipient, transformation was reduced only to about 10%. Orrego et al. (259) envisioned the process of DNA transfer due to contact between the outgrowing spore and the recipient competent cell, the latter perhaps providing, by its autolysin activity, access to the cell wall-associated DNA for uptake. The authors believed that this type of gene transfer, which in its phenomenology is reminiscent of conjugation, could also occur in nature since competence is developed during outgrowth of germinating spores (332).

A means of providing such a tight physical contact between cells is by their concentration on membrane filters. Several lines of evidence point to transformation as the probable mechanism of transfer of chromosomal markers in "matings" between *H. influenzae* strains on filters or agar surfaces (4). First, the transfer occurred in the absence of plasmids and, like chromosomal transformations with free DNA, was dependent on *rec* functions of the recipient. Second, the transfer was sensitive to DNase. DNase I at 125 μg ml^{-1} reduced the frequency by about 1 order of magnitude while abolishing transformation by DNA extracted from cells. Also, the transfer was not affected by competing homologous DNA as opposed to transformation by free DNA. Thus, there seems to be relatively tight contact between exchanging cells. Third, cell-to-cell chromosomal, but not conjugative plasmid transfer, was stimulated when recipients in matings were competent for uptake of free DNA.

Albritton et al. (4) hypothesized that it was not DNA released from lysing *H. influenzae* cells that was the source of transforming activity in cell contact-mediated transformation but, rather, DNA provided by some other mode of externalization. By using an experimental system similar to the *H. influenzae* system, an active role for donors in cell contact transfer of chromosomal genes was demonstrated in *P. stutzeri* (345). The transfer was inhibited by the replication inhibitor nalidixic acid in "matings" of nalidixic acid-sensitive donors and resistant recipients. In this organism, DNase-sensitive transfer of markers was about 1,000 times more frequent in filter "matings" than in standard transformations with extracted DNA and recipient cells in liquid medium.

Highly DNase I-sensitive interspecific transfer of a nonconjugative plasmid was observed within the genus *Streptococcus* (34). Among several recipients tested (*S. pyogenes*, *S. faecalis*, *S. sanguis*, *Staphylococcus aureus*, *Listeria inocua*), transfer occurred only from *S. pyogenes* to the highly transformable *S. sanguis* Challis strain and only under conditions of cell contact in filter matings. Attempts to transfer the plasmid from *S. sanguis* to the other strains were unsuccessful. Transformants were verified by phenotypic analysis and the physical presence of the plasmid. Transformation also occurred in a DNase-sensitive manner from killed *S. pyogenes* donors (34). Phages were not detected in cell-free filtrates of either untreated or UV- or mitomycin C-treated donor cultures. The plasmid

DNA seemed to be associated with the exterior of the donor cells, since no transforming activity was found in the supernatants of chloroform-killed donors. The data collectively argue for a transformation-mediated cell-cell contact-dependent plasmid transfer.

In recent work, Paul et al. (282) showed the transfer of several nonconjugative broad-host-range plasmids in the absence of transmissible helper plasmids from *E. coli* to a naturally transformable *Vibrio* sp. Similar transfer frequencies were obtained in liquid and filter matings. The transfer was sensitive to DNase and occurred from viable as well as from heat-killed *E. coli* donors to the *Vibrio* sp. Transformation by plasmids did not occur when donors and recipients were separated by a filter (pore size, 0.2 μm), indicating a requirement for cell contact.

As a general conclusion, during cell contact, chromosomal and plasmid genes may be transmitted in a way that appears to have more features of transformation than of conjugation. Interspecies transfer of nonconjugative plasmids by natural transformation, be it via cell-cell contact or via free DNA, could be a potent alternative to mobilization by transmissible plasmids. Further examples are discussed below (see the section on interspecies plasmid transformation).

Fate of Internalized DNA

When considering that a competent cell in the environment would take up DNA originating from various other organisms present in the same habitat, it is important to determine what influences the integration of the internalized DNA into the recipient genome. When single-stranded donor DNA is assimilated, single mismatched bases or stretches of unpaired bases may result from allelic differences between donor and recipient. What is the response of the recipient cell? In the following sections, this will be discussed in some detail. Further, examples of the interspecific transfer of genes by transformation will be provided.

Homology and heteroduplex formation. Recombination between homologous DNA segments depends, in *E. coli* and other bacteria, on the *recA*$^+$ gene (212). Genetic transformation by chromosomal DNA is abolished in *recA* mutants of *S. pneumoniae*, *B. subtilis*, *H. influenzae*, *P. stutzeri*, and *Acinetobacter calcoaceticus* (272, 325, 383). In *E. coli* the minimum length of a homologous region between two otherwise nonhomologous duplex circular molecules allowing recombination was measured by plasmid-phage cointegration in vivo and found to be 20 to 40 nucleotide pairs (168, 317). Shen and Huang (317) observed a sharp decline in the number of recombinants with decreasing homology in that region. For instance, compared with 100% homology in a region of about 400 bp, a 42- and 300-fold decrease of cointegrate formation was observed with 90% and 65 to 70% of shared homology, respectively. Since the frequencies of recombinants resembled those for short, perfect homologies, it was concluded that initiation of recombination by RecA protein between heterologous sequences depends on regions of perfectly paired bases. Heterologous DNA, internalized by competent *B. subtilis* cells, seems to become associated with the recipient chromosome in an unstable form via stretches of paired bases (362). On the basis of the findings that 90% of donor material in the heteroduplex was sensitive to digestion by single strand-specific DNase (S1 nuclease) and that the donor-recipient complex had a melting temperature of only 48°C, te Riele and Venema (364) supposed that the labile association between the heterologous donor strand and the recipient chromosome is mediated either by various short regions of 15 to 25 matched base pairs or by an extended stretch of about 200 nucleotides, including 25% mismatched bases. Recent data show that homologous recombination between a plasmid and the *B. subtilis* chromosome required at least approximately 70 bp of homology (167). te Riele and Venema (364) proposed a RecA-catalyzed strand exchange mechanism in *B. subtilis* which is similar to that proposed for *E. coli*. This proceeds by initiation of donor-recipient complex formation at short homologous sequences followed by RecA protein-mediated branch migration into heterologous regions. It should be noted that the physical stability of an initial heteroduplex joint depends not only on its length and the degree of homology between the paired strands but also on the action of enzymes which would dissociate such a joint. Thus, such enzymes would act as a recombinational proofreading device, as recently suggested when the antipairing activity of DNA helicase II (UvrD protein of *E. coli*) was detected in vitro (241).

Mismatch correction. Nonmatching nucleotide pairs within a stretch of perfectly paired nucleotides are termed mismatches or mismatched base pairs. Assimilation of the donor single strand into the recipient genome leads to the formation of mismatches as a consequence of nucleotide exchange or deletion/insertion mutations in the donor or recipient DNA. Mismatches can be subject to enzymatic correction in the cell. With respect to transformation, this has been well documented in *S. pneumoniae* with the help of *hex* mutants which are deficient in mismatch correction. The current understanding of mismatch correction during transformation will be summarized briefly. An extensive review on this subject has been provided by Claverys and Lacks (48). *hex* mutants have also been identified in *H. influenzae* (11).

Marker-specific transformation efficiencies, as defined by the number of transformants of a specific donor marker in relation to that of a reference donor marker, may vary 20-fold among different markers in *S. pneumoniae*. The transformation efficiency of single-site mutations in donor DNA may fall into one of essentially three classes: high-efficiency markers (HE; relative transformation efficiency around 1.0), low-efficiency markers (LE; 0.1 and less) and intermediate-efficiency markers (IE). Gasc et al. (97) studied the correction of mismatches in the *amiA* locus, which determines resistance to amethopterin. The effect of insertions of 1 or 2 bases is similar to that of transition mutations. These transform as IE markers. Increasing the nonhomology in the donor DNA sequence (4, 5, and 34 bp in the form of deletions or insertions) progressively increased the transformation efficiency (IE to HE marker). Apparently, the mismatch correction system (Hex) is restricted to the recognition and correction of single-base mismatches and nonhomologous regions of only up to 3 bp. The correction process seems to involve long patches of excised DNA as evidenced by the decrease of the transformation efficiency of HE markers when adjacent to LE markers and the physical loss of donor DNA fragments, containing an LE marker, from the heteroduplex (48). In more recent studies, Gasc et al. (98) examined 24 single-base-pair mismatches, formed during transformation, for their susceptibility to the Hex correction system. It was found that among these, several performed as LE markers (e.g., G · T, A · C, G · G), whereas others behaved as IE markers (T · T, A · G) or were not corrected at all (HE marker; C · C). The flanking nucleotide sequences affected the correction of some mismatches. The data provided information on the specificity of the Hex mismatch recognition system for single mismatches. The studies led to the idea that the efficiently recognized mismatches are those which can adopt an intrahelical form, whereas the poorly recognized mismatches (HE markers) would be those with a looped-out structure (98).

This would be consonant with the low recognition of multiple-base-pair mismatches (insertions, deletions [97]). Since *hex* mutants of *S. pneumoniae* and *H. influenzae* have a mutator phenotype (high spontaneous mutation frequencies [48]), it is concluded that the Hex system is involved in the correction of (mostly single-base-pair) replication errors, as is the *E. coli* Mut system (48, 238). Once a mismatch is recognized in the heteroduplex DNA during transformation, what directs the Hex system to correct against the donor DNA, thereby making the marker an LE marker? Among the possibilities considered, one relied on methyl-directed strand selection. In *E. coli* (and *Salmonella typhimurium*), retarded methylation of a newly synthesized strand during replication directs mismatch correction by the Mut system (encoded by *mutH*, *mutS*, *mutL*, and *dam*) toward conservation of parental information (238). The new nonmethylated strand is excised, and repair synthesis complementary to the parental DNA restores the genetic information in a conservative fashion. A difference in the degree of strand methylation could explain Hex-dependent removal of LE markers in *S. pneumoniae* only if demethylation of the donor DNA strand occurred during entry into the recipient cell. However, demethylation of naturally occurring methylated sites in DNA has not been demonstrated so far (48). A more plausible explanation considers that during integration of the donor single strand into the recipient genome, two strand interruptions will flank the donor single strand in the heteroduplex region before the covalent linking of donor with recipient DNA occurs by ligation. These strand interruptions could serve as the entry sites for the Hex-associated excision enzyme, removing the assimilated donor strand carrying the LE marker (121). Accordingly, single-strand interruptions at the replication fork would allow entry of the elimination of mismatches produced during replication, thus leading to avoidance of mutations (48). Data obtained with *S. pneumoniae* suggest that specifically long stretches of heterology, which may be formed in heteroduplex DNA following transformation or as replication errors and which are not recognized by the Hex system, can be corrected by specific, DNA polymerase A-independent conversion (184).

The fact that interspecies transformation frequencies with chromosomal markers are low (see the next section) (Fig. 2) suggests that extensive mismatch correction along with donor DNA exclusion from heteroduplex is operative. In conjugative chromosomal DNA transfer between *E. coli* and *Salmonella typhimurium*, the mismatch correction system (Mut) effectively reduced gene transfer. This was concluded from the finding that the number of exconjugative recombinants increased up to 1,000-fold when the recipient was defective in the Mut system (290). The gross DNA sequence homology between *E. coli* and *Salmonella typhimurium* is about 80% (254). Similarly, the transformation frequency of an *S. pneumoniae hex* mutant indicated loss of the discrimination between LE and HE markers so that they all resembled HE markers (176).

Interspecies chromosomal transformation. In the preceding sections we discussed the requirement for a minimum of homology for the formation of the donor-recipient DNA complex and we determined that the type of mismatch and, at least in some cases, the surrounding sequence can have a profound effect on the persistence in the recipient of a marker in the donor molecule. Various studies provide evidence that transfer of chromosomal genes by transformation can occur across species and even higher taxonomic boundaries (Fig. 2).

The interspecies passage of chromosomal genes is limited mostly to members of the same genus (Fig. 2). We have included in Fig. 2 the available transformation efficiencies of donor DNA from several species for the transformable recipient. In the genus *Haemophilus*, for instance, the transforming efficiency of the streptomycin resistance markers, reflecting mutations in a ribosomal protein gene, showed a 25,000-fold variance among the strains tested. The efficiency of transformation of *H. influenzae* by DNA of nine different *Haemophilus* species (Fig. 2) did not indicate a correlation to the degree of nucleotide sequence heterology (determined by gross DNA hybridization) and the ability to take up the heterospecific DNA as a result of the presence of a recognition sequence in the DNA homologous to that of *H. influenzae* (apparently present in six of the nine species [5]). Albritton et al. (5) concluded that local nucleotide sequence divergence, not detectable by hybridization, affected the efficiency of heterospecific transformation in *Haemophilus* spp. It is conceivable that the interspecific transformation occurs preferentially at conserved loci. For example, Harford and Mergeay (125) showed that rifampin and erythromycin resistance markers from various *Bacillus* species transformed *B. subtilis*, albeit with several orders of magnitude variation of the efficiency among the donors (Fig. 2). In contrast, presumably nonconserved markers (auxotrophy) were transferred only by three donor strains and with lower efficiencies for two of them (2- and 95-fold) than of the transfer of the resistance markers (125). The determinants of nitrogenase (*nif*) are another example of conserved sequences. The *nif* genes and the *nif* operon structures are conserved among the various diazotrophic bacteria examined (e.g., *K. pneumoniae*, *Rhizobium meliloti*, *Azotobacter vinelandii*, *Anabaena* sp.), but the arrangement of the operons on the chromosome or plasmids differs considerably (128, 165). Nif^+ transformants of *nif* mutants of *Azotobacter vinelandii* were obtained with DNA from a variety of nitrogen-fixing species, including the same genus (Fig. 2). The transformation efficiencies (transformation frequencies obtained with heterologous nif^+ DNA relative to those obtained with DNA of a nif^+ strain of *Azotobacter vinelandii*) declined in the order *Azotobacter chroococcum* (21%), *Azotobacter paspali* (2%), *Azotobacter beijerinckii* (0.3%) (74, 263). Interestingly, donor DNA (nif^+) from *R. meliloti* and *Beijerinckia indica*, both of which belong to the gamma subclass of the proteobacteria and therefore are distantly related to *Azotobacter vinelandii* (alpha subclass [70]), transformed *Azotobacter vinelandii* to the Nif^+ phenotype with a low frequency of 0.01 and 0.002%, respectively (23, 263), whereas with nif^+ DNA from *Azomonas* spp., which are in the same family as *Azotobacter vinelandii* (70), no transformants were obtained. Thus, and similar to the situation for *Haemophilus* spp., there is a lack of correlation between the relatedness of the donor to the recipient and the transformation efficiency of the conserved marker. The reason for this is not known. In an attempt to develop a genetic system for the study of the symbiosis of *Rhizobium* spp. and leguminous plants, Bishop et al. (23) transformed *Azotobacter vinelandii* with *R. trifolii* DNA to nitrogen fixation proficiency (Fig. 2). Among 46 Nif^+ transformants, 6 were identified to carry the additional characteristic of *R. trifolii* to be agglutinated by trifoliin and an antiserum to clover root antigen. Immunofluorescence assays supported the conclusion that the cell surface of *Azotobacter vinelandii* transformants had changed to cross-react with the root wall lectin trifoliin. Nodulation of white clover roots, however, could not be demonstrated. It should be noted that genes for symbiosis and for several determinants of nitrogen fixation in *Rhizobium* spp. are on megaplasmids (189). Bishop et al. (23) did not find evidence in *Azotobacter vinelandii* transformants for close linkage of nitrogen fixation proficiency and trifoliin agglutination ability and for the presence of a plasmid, suggesting that plasmid genes had been integrated in the *Azotobacter vinelandii*

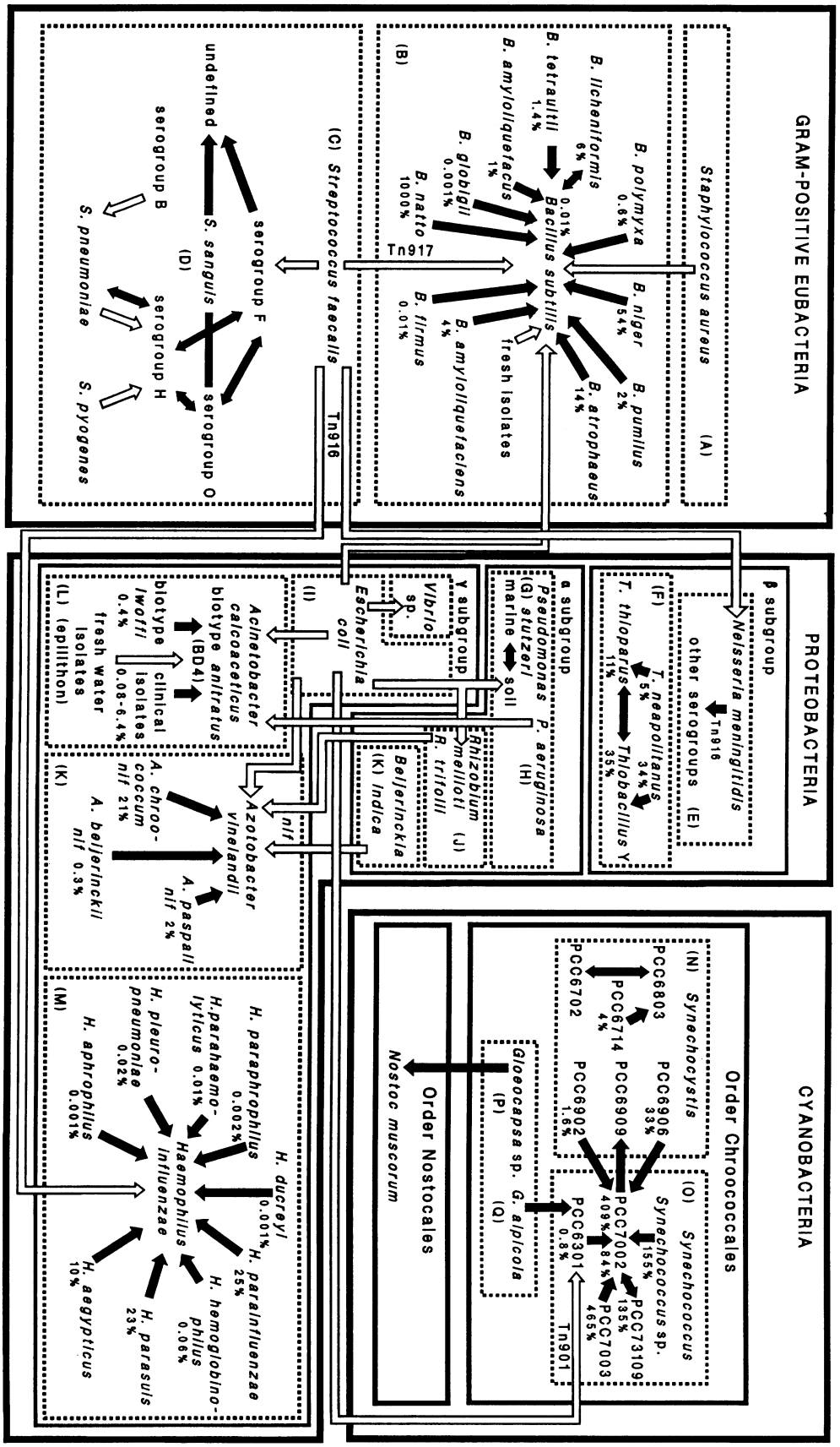

FIG. 2. Routes of gene transfer by transformation among bacteria. The arrows indicate in vitro transfer of chromosomal markers (solid arrows) or of plasmid and (as indicated) transposon markers (open arrows). Values below species names or strain designations indicate the interspecies or interracial transformation frequency relative to that obtained when the recipient was transformed by homologous DNA. Letters in brackets refer to the references as follows: (A), 120; (B), 82, 102, 120, 125, 127, 214, 221, 363; (C), 163, 164, 398; (D), 34, 46, 183, 283, 288, 308; (E), 163; (F), 394; (G), 348; (H), 237; (I), 32, 59, 62, 73, 99, 173, 198, 237, 282, 294, 321, 379; (J), 23, 263; (K), 263; (L), 20, 153, 298; (M), 5; (N), 115, 342; (O), 342; (P), 322; (Q), 71.

chromosome. Figure 2 depicts a variety of detected transfer routes of chromosomal DNA by transformation between different species, genera, and orders.

Interspecies plasmid transformation. The transfer of plasmids between cells of different species requires broad-host-range replication initiation of the plasmid. The reconstitution of a plasmid or other autonomously replicating genetic elements after uptake of single-stranded portions during transformation does not require homology to the recipient DNA per se (see the section on biology of natural genetic transformation, above). The reconstitution of a plasmid from internalized single-stranded fragments may, however, require recombinational or repair functions of the host cell. For instance, the transformation of a *recA* mutant of *P. stutzeri*, otherwise isogenic to the wild type, by plasmid DNA was about 10% of the efficiency of the *recA*$^+$ strain (16). Despite such requirements and different restriction systems present in cells from different species (see barriers to transformation, below), broad-host-range plasmids and transposons may pass between all major branches of the prokaryotic kingdom (Fig. 2).

Transformation In Situ

Experiments for studying genetic transformation in bacterial habitats other than inside animals, such as soil, sediment, and water, were performed mostly in microcosms, containing material sampled from the environment. The material was used sterilized or nonsterile to differentiate between biological and physicochemical parameters influencing transformation. The experimental system generally included transforming DNA and a naturally transformable recipient organism either obtained from a culture collection or isolated from the particular site of sampling. It has proven advantageous for the interpretation of results from the microcosm experiments to use bacterial strains that have been characterized with respect to the physiology of transformation. Most of the studies deal with the ability of the strains introduced into the microcosm to develop competence, to take up DNA, and to express selectable markers acquired in the microcosm. As a result of numerous investigations summarized in the following sections, it appears that there is no specific model organism that by its properties is representative of other naturally transformable bacteria in a given habitat.

Aquatic environments. Most transformation experiments in microcosms have been reported for the marine environment. The organisms used were the marine isolates *Vibrio* strain DI9 or *Vibrio* strain WJT-1C, a strain that has been isolated as a spontaneous high-frequency-of-transformation mutant of DI9 (93), *Vibrio parahaemolyticus* (347), and the stock culture strain *P. stutzeri* ZoBell, which is a marine close relative to the *P. stutzeri* soil strain (347). Several physiological features of the transformation systems of *Vibrio* spp. and the *P. stutzeri* soil strain have been studied (32, 93, 94, 201, 346).

Stewart and Sinigalliano (347) examined transformation of *P. stutzeri* ZoBell in the marine sediment habitat. Stationary-phase cells suspended in artificial (sterile) seawater were added to microcosms with sterile sediment and incubated overnight in the presence of homologous chromosomal DNA with a rifampin resistance marker. The 11-fold higher frequency of rifampin-resistant cells over the background suggested a low level of transformation. The frequency of transformation increased from 10^{-7} to 10^{-6} with increasing amounts of DNA (from 1 to 3 μg cm of sediment^{-3}) in the sediment. No transformants were found when DNase I was added. Transformants were found only among cells associated with the sediment particles. It is not known whether transformants were due to uptake of sediment-adsorbed DNA by competent cells of *P. stutzeri* ZoBell or whether the DNA molecules were first released from the surfaces of sediment particles and then taken up. Detailed studies with the *P. stutzeri* soil isolate and with other bacteria, in purified sediment and other natural material, clearly demonstrated that transformation can proceed via DNA adsorbed on minerals (42, 196, 201, 302). The DNA concentration at which transformation of *P. stutzeri* ZoBell in sediment was saturated (0.6 μg cm of sediment^{-3}) was reduced to about one-third when calf thymus DNA (10 μg cm of sediment^{-3}) was preloaded on the microcosm (349) (*P. stutzeri* takes up DNA of its own species selectively [32, 38, 201]). This suggested that not all of the *P. stutzeri* DNA added was available for transformation unless sites not accessible to competent cells were saturated with calf thymus DNA. A variety of other sterilized freshwater and marine sediments gave low frequencies of Rif^r transformants (between 8.2×10^{-7} and 5.7×10^{-6} [347]). Transformation at the same level was noted in a nonsterile marine sediment microcosm. In contrast, transformation of the *P. stutzeri* soil isolate, *V. parahaemolyticus*, and *Vibrio* strain DI9 was hardly detectable even in sterile sediments (347).

The unequivocal verification of the genotype of transformed cells by means of physical detection methods such as DNA-DNA hybridization is desirable when selecting transformants against the background of the ambient microbial population. Transformation with plasmid DNA offers a way (DNA hybridization) to differentiate real transformants from naturally resistant autochthonous bacteria. Paul et al. (278) investigated transformation of *Vibrio* strain WJT-1C in marine water and sediment with a multimeric form of the Tn*5* insertion derivative of plasmid R1162 (RSF1010). The result was that plasmid transformation occurred at low frequency in sterilized samples of water and sediment from various marine sites but occurred in nonsterile microcosms only in water. Nutrient amendment did not give rise to transformants in nonsterile marine sediments but enhanced the number of transformants in nonsterile water almost 40-fold. This amendment was considered (278) to enable *Vibrio* spp. to reach the stationary phase and thus to attain maximum competence (94). Paul et al. (278) assumed that transformation of *Vibrio* spp. may occur in the marine environment but with higher probability in the water column than in the sediment and with higher probability at estuarine locations receiving increased nutrient input than in offshore waters. The failure to detect plasmid transformants in nonsterilized sediment material was speculated to be due to a high level of microorganisms, producing extracellular DNases. Alternatively, the sediments may contain metabolism-inhibiting compounds such as sulfides (278) or substances that directly inhibit competence development or transformation.

The results of the transformation studies of marine sediments are contradictory. Perhaps this reflects the impossibility of generalizing results obtained with an ecotype of a bacterial species and its environment. Clearly, more data must be collected to gain some insight in the processes and parameters governing transformation in the aquatic environment. The importance of the transportation of bacterial cells and their DNA (free, adsorbed, or packaged in phages) in habitats such as water, sediment, and plant material for horizontal gene exchange has been emphasized recently (185).

Terrestrial environments. Graham and Istock (107) monitored the exchange of chromosomal genes by transformation between mixed laboratory strains of *B. subtilis* in sterile soil. The strains did not have plasmids or generalized transducing phages. Each strain contained a block of three linked markers lying on opposite sides of the genetic map. To facilitate the

identification of recipient cells and the routes of gene passage, each strain had been additionally marked by two different resistance determinants one of them not cotransferred with the linkage block. Heat-activated spores of each of the two strains were washed into autoclaved potting soil. The growth of the parents and the appearance of recombinants under nonselective conditions (i.e., with addition of amino acids supplementing auxotrophies) were monitored for 8 days. The mixed-strain soil culture showed relatively high exchange frequencies (1.8×10^{-4}) of the linkage blocks in both directions after 24 h. Thereafter, the frequencies progressively declined. A striking observation was that the mixed-strain soil culture reached a titer significantly higher than that of single-strain cultures in soil. The authors concluded that two-way exchange of genes effected ecological adaptation. The decline of the number of triple recombinants could be explained by the finding that certain markers were selected for while others were selected against. For instance, in one parent the *his*, *trp*, and 3-aminotyrosine resistance (AMT^r) markers were closely linked whereas the 4-azaleucine resistance marker (AZL^r) was not cotransferred. In the soil culture, among selected recombinants for other resistance markers, the *his trp* genotype rose during 4 days and then remained constant for the next 4 days, in contrast to the AMT^r and AZL^r genotypes, which continuously decreased. On day 8, the most common phenotype in soil was $His^- Trp^-$, having in addition three of the four markers of the second parental strain (for detailed information on the kinetics of the population structure and evolution of the recombinant genotypes, see reference 108). Moreover, both parental genotypes were totally lost within 1 day in soil. Similar results were obtained in other experiments with *B. subtilis* in which different inoculum sizes, ratios of inoculum strains, and nutritional conditions were used (109). When the sterile soil was inoculated with single strains and transforming DNA, high frequencies of transformants (several percent) were found (107). Interestingly, neither addition of a large excess of calf thymus DNA (known to decrease transformation in vitro by competition with homologous DNA for uptake) to the microcosm nor addition of large amounts of DNase reduced the frequencies of triple recombinants in this experiment. More recent microcosm experiments may give an explanation of the latter finding. DNA adsorbed on mineral particles and other solids in soil is less available for enzymatic degradation than free DNA (see the section on fate of extracellular DNA in the environment, above) while still available for uptake by competent cells (see below). Graham and Istock (107, 108) interpreted their results in the soil microcosm to mean that an evolutionary shift of the gene pool within the population had occurred as the result of exchange of genes by transformation.

Spontaneous interspecific transfer by transformation of chromosomal markers between *B. subtilis* and *B. licheniformis* in sterile soil has also been reported to occur in both directions (85; for a review, see reference 147). Unlike the intraspecific exchange of markers in soil culture crosses of *B. subtilis* (107), hybrids between *B. subtilis* and *B. licheniformis* had unstable phenotypes. Duncan et al. (85) argue that recombination does not disturb overall distinctness of the two species but could erase local distinctness.

In reciprocal transformational crosses of desert soil isolates and the standard *B. subtilis* 168 Marburg and between the isolates themselves, 6 of 24 strains were able to act as a donor or recipient but the transformation frequencies were low (51). Cohan et al. (51) and Roberts and Cohan (296) were able to attribute the low transformability of some strains by DNA of other strains (which they called sexual isolation in the natural population of *B. subtilis*) to the low competence of the recipients, to the action of restriction enzymes in the recipients, and to sequence divergence. Such barriers to transformation are discussed in more detail in the next section. Investigations of allozyme variation, phage and antibiotic resistance, and restriction fragment length polymorphism in soil isolates of *B. subtilis* each support the view that genetic exchange in natural *B. subtilis* populations must be relatively frequent (84, 148).

How could the transfer of genes by free DNA proceed in soil? This question was addressed by a series of experiments involving *Acinetobacter calcoaceticus*, a useful model organism for ubiquitous soil and water bacteria. *Acinetobacter calcoaceticus* is transformable in groundwater and soil liquid, even in the presence of indigenous microorganisms, with similar efficiency to that in vitro (198). The concept of the experiments of Chamier et al. (42) was to examine whether chromosomal and plasmid DNA adsorbed on the "dirty" mineral material sampled from a groundwater aquifer was available for transformation. Chromosomal DNA tightly bound in the microcosm was readily taken up with frequencies of transformants only slightly lower than with dissolved DNA or DNA adsorbed on purified sand. Evidence was presented that transformation occurred mainly in the solid/liquid interface and not by DNA desorption from the minerals during the experiment. In contrast, essentially no transformation was found when large amounts of monomeric plasmid DNA were adsorbed on sand (as a soil model) and groundwater aquifer material. However, the simultaneous presence of chromosomal and plasmid DNA on the minerals produced plasmid transformants of *Acinetobacter calcoaceticus*, although the frequency was still 3 orders of magnitude lower than that obtained with free plasmid DNA in solution (42). A similar observation was made by using the groundwater aquifer microcosm and *B. subtilis* and DNA of a plasmid carrying a *B. subtilis* chromosomal insert (207, 302). Several possible explanations for the specific decrease of plasmid transformation on minerals were considered by Chamier et al. (42) and Romanowski et al. (302): (i) circular plasmid molecules adsorb to sites, which may be different from those where linear DNA binds on the mineral material, and thereby would not be accessible to competent cells; (ii) plasmids bind too tightly to be detached by cells during uptake; (iii) the introduction of double-strand breaks upon adsorption on minerals would destroy the transforming activity of plasmids; and (iv) the three-hit dependence of mineral-adsorbed plasmid DNA transformation in *Acinetobacter calcoaceticus* (in addition to immobilization of the DNA), in contrast to the two-hit kinetics of transformation by dissolved DNA, reduces the chance of productive collisions between cells and DNA molecules at the solid/liquid interface. Further experimentation is required to distinguish among these possibilities and to explain the helper effect of chromosomal DNA for the transformation by plasmid DNA. Apart from this, the microcosm experiments with the gram-positive (*B. subtilis*) and gram-negative (*Acinetobacter calcoaceticus*) soil bacteria suggest that inorganic precipitates (e.g., iron and manganese oxyhydroxides) and other deposits on the natural groundwater aquifer (and possibly soil) minerals do not hamper transformation by chromosomal DNA. Plasmid DNA, when associated with particulate material, may have a lower probability of transforming cells than dissolved plasmid DNA has.

Figure 3 shows a scheme of the flow of genes by transformation, which is proposed on the grounds of microcosm experiments to occur in aquatic and terrestrial habitats (193). Free DNA is produced continuously by cellular lysis and excretion, leading to a pool of extracellular DNA (see the section on free DNA in the environment, above). The released DNA is distributed between the liquid phase and surfaces of

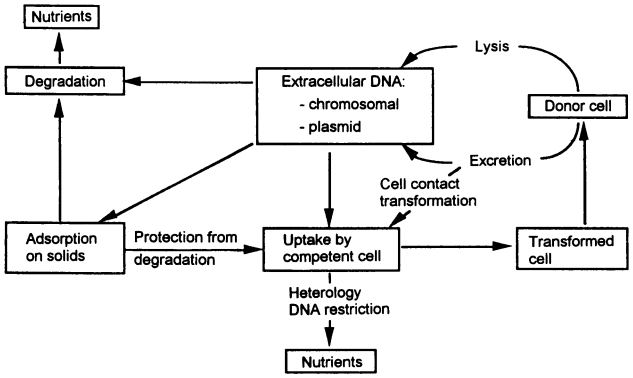

FIG. 3. Scheme of gene transfer by free DNA in the aquatic and terrestrial environment. Modified from reference 193 with permission of the publisher.

minerals and other solids in soil and sediment and of suspended particulate material in aqueous habitats (see the section on fate of extracellular DNA in the environment, above). This extracellular DNA may transform competent cells. On the other hand, free DNA or DNA taken up by cells but not inheritably integrated (because of lack of homology or DNA restriction [see the sections on fate of internalized DNA, above, and DNA restriction, below]) is degraded by extracellular and intracellular DNases, respectively, and the products are used as nutrients (see the section on biological functions of DNA uptake other than gene acquisition, below).

Habitat of pathogenic bacteria. It was an in vivo experiment that first demonstrated transfer of bacterial genes by transformation. In 1928, Griffith (114) observed that mice were killed when infected with a mixture of heat-killed pathogenic S-form ("smooth") and living nonpathogenic R-form ("rough") *S. pneumoniae* cells. Pneumococci isolated from the cadavers revealed the S-form colony type. Griffith (114) concluded that the R-form had undergone transformation by the dead S-form, not knowing at that time what substance had caused this morphological change. In 1944, Avery et al. (10) identified DNA as the transforming principle in Griffith's experiment.

There is no other direct experimental evidence for genetic transformation in vivo. Nevertheless, results of cocultivation experiments suggest that substantial intra- and interspecies transfer of virulence determinants via transformation occurs in *Neisseria* spp. (94a). An increasing body of nucleotide sequence data of chromosomal genes in transformable species further suggests the occurrence of frequent genetic exchange within and between species. Pathogenic bacteria are attractive objects of research because clinical isolates from various geographical locations and different periods of sampling are readily at hand and can be used to trace the emergence of specific genotypic variations. For instance, the frequent incidence of low-level resistance against penicillin in pathogenic bacteria has prompted extensive research on its basis and the origin of the resistance. In several *Neisseria*, *Streptococcus*, *Staphylococcus*, and *Haemophilus* clinical isolates (see reference 77 and references therein), the resistance was the result of altered penicillin-binding proteins. Nucleotide sequence comparisons of the genes of sensitive and resistant isolates revealed intragenic sequence blocks with a high degree of divergence. For *N. gonorrhoeae* and *N. meningitidis*, these regions appear to originate from *N. flavescens*, a commensal colonizing the same habitat, the nasopharynx, as *N. meningitidis* (334). The hybrid genes of the two pathogens seem to have formed by replacement of homologous sequences with corresponding regions of the *N. flavescens* chromosome. A similar recruitment of foreign genes or gene segments, although the origin has not been identified yet, is claimed for the emergence of the mosaic structure of penicillin-binding protein genes of penicillin-resistant clinical isolates of *S. pneumoniae* (77), which presumably transferred the genes to other streptococci (50, 78). Dowson et al. (77) underlined the fact that the emergence of altered penicillin-binding proteins occurred in species known to be naturally transformable. The potential for transformation seems to be high in natural populations. Surveys of clinical isolates of *S. pneumoniae* (395) and *H. influenzae* (305) showed that 6 of 9 (66%) and 13 out of 31 strains (42%), respectively, were transformable.

Other habitats. There may also be other potent habitats for gene exchange by transformation. For instance, root nodules contain high titers of bacteroid rhizobacteria, which may develop natural competence and transport DNA (released from some cells) actively into their cytoplasm or internalize DNA by a passive uptake event (see Introduction). Other habitats for high bacterial concentrations, which may favor transformation by free DNA or during cell contact, are the intestines of insects, worms, and warm-blooded animals, the interior of protozoa (after ingestion), and the surface, mesophyll, and intracellular space of plants.

Estimation of Transformation Frequencies in the Environment

The microcosm experiments discussed in this section suggest that transformation occurs in several microbial habitats. When one compares the transformation frequencies determined in various experimental systems, simulating natural habitats, with those obtained under optimized conditions in appropriate media, both small and large differences are apparent (Table 5). The differences between transformation frequencies observed in microcosms and those actually occurring in an undisturbed habitat may vary in the same range. This is because, for instance, environmental parameters (such as temperature, pH, nutrient level, and nutrient flux), the number of microorganisms, and the composition of the population as well as barriers to transformation (see below) may not be accurately simulated in the microcosm. Extrapolation of transformation frequencies from microcosms to the environment could be misleading because concentrations of transforming DNA in situ are not known (and must reach certain levels depending on the recipient cell concentration to make DNA-cell collisions likely). Furthermore, the specific transformation characteristics of a species in its habitat are probably far from being fully known. However, the data in Table 5 suggest that transformation in the environment can be quite frequent, at least occasionally.

BARRIERS TO TRANSFORMATION

When considering quantitative data available from physiological and genetic studies as well as from microcosm experiments, it becomes apparent that transformation frequencies differ considerably among the species examined (Tables 1 and 5). In this section some factors, intrinsic to the cells and to the environment, that may limit the extent of transformation in natural bacterial ecosystems will be discussed.

Cellular Level

DNA restriction. As Goodgal (104) has pointed out in his review, transformation of *B. subtilis*, *S. pneumoniae*, and *Haemophilus* spp. by chromosomal markers seems not to be

TABLE 5. Transformation under standard laboratory conditions and under environment-simulating conditions

Recipient organism (transforming DNA)	Transformation frequency in:		Environmental sample in microcosm	Reference(s)
	Medium	Microcosm		
P. stutzeri ZoBell (chromosomal)	5.1×10^{-5}	1.2×10^{-6}	Nonsterile marine sediment	347, 348
P. stutzeri soil strain (chromosomal)	7.0×10^{-5}[a]	2.9×10^{-4}	Nutrient-amended soil extract	203
Acinetobacter calcoaceticus (plasmid)	9.8×10^{-4}	3.9×10^{-4}	Nonsterile groundwater and extract of fresh soil	198
A. calcoaceticus (chromosomal)	7.0×10^{-3}[a]	2.6×10^{-4}	Groundwater aquifer material (sterile)	42
B. subtilis (chromosomal)	3.5×10^{-2}[a]	3.5×10^{-6}	Groundwater aquifer material (sterile)	302
Vibrio sp. (plasmid)	2.5×10^{-4}[a]	2.7×10^{-10}	Nonsterile marine water	278
Concentrates of unknown microbial assemblages[b] (plasmid)	1.1×10^{-9}–3.6×10^{-6}			92

[a] For references, see Table 1.
[b] From marine water, sponges, or sea cucumber gut.

affected by the presence of a restriction system in the recipient. For instance, Harris-Warrick and Lederberg (127) obtained prototrophic transformants of *B. subtilis* (*trp tyr*) with DNA from a prototrophic *B. globigii* strain at a very low frequency. The reason could be DNA restriction (the two strains were shown to have different modification and restriction systems) or nucleotide sequence divergence. To distinguish between these possibilities, a UV light-induced mutant (*trp tyr*) was isolated from the prototrophic transformant. This was transformed to prototrophy by DNA from *B. globigii* (and *B. subtilis*) several orders of magnitude more frequently, indicating that sequence divergence and not DNA restriction was the barrier for this interspecies transformation. In *S. pneumoniae*, transformation of strains with different restriction systems by chromosomal DNA occurred with identical efficiency (180). Although these results indicate that DNA restriction has no influence on chromosomal transformation, more recent results of crosses between certain soil isolates of *B. subtilis* and the standard 168 Marburg strain suggest that DNA restriction may play some (although not a great) role as a barrier for transformation in natural populations. This, besides sequence divergence and variations in competence (see below), can lead to sexual isolation within this species (51).

Transformation of *N. gonorrhoeae* by plasmid DNA isolated from *E. coli* was 5 orders of magnitude less efficient than transformation by plasmid DNA isolated from the same strain (338, 339). A less dramatic restriction was noted for plasmid transformation of *P. stutzeri*, reducing transformation by shuttle vector plasmid DNA isolated from *E. coli* instead of *P. stutzeri* by 1 to 2 orders of magnitude (32, 39). *Anacystis nidulans* (318) and *Acinetobacter calcoaceticus* (198) were similarly well transformed by plasmid DNA extracted from the same strain or from *E. coli*. These data do not provide a general picture of restriction as a barrier for transformation by plasmid DNA. Perhaps bacteria have different strengths of restriction systems, such as in various soil isolates of *B. subtilis* (296, 378). On the other hand, transformation by plasmid DNA may, in the recipient, involve intermediates which consist of single-stranded and duplex regions during plasmid reconstitution (see the section on reconstitution of plasmid DNA molecules, above). Mechanisms of plasmid reconstitution can be species specific. Intermediate structures, particularly duplex regions consisting of two donor DNA strands, would be targets for restriction enzymes if the methylation pattern of the donor DNA does not match that of the recipient. Clearly, more experimentation is necessary to find the reasons why DNA restriction is a barrier to transformation in one instance and not (or hardly) in another.

Sequence divergence. The cellular functions for the process of DNA uptake and the integration of single-stranded donor DNA material in the recipient genome have been described above (see the sections on biology of natural genetic transformation and transformation in the environment). Roberts and Cohan (296) examined the influence of sequence divergence on the transformation in *B. subtilis* by studying the transfer of the highly conserved *rpoB* locus (rifampin resistance marker, originating from a mutation in this gene which codes for the β subunit of RNA polymerase). For this purpose, a nonrestricting Marburg strain was transformed by genomic DNA from a rifampin-resistant Marburg strain (as a reference) and by PCR-amplified *rpoB* DNA from rifampin-resistant mutants of various *B. subtilis* soil isolates and other laboratory *Bacillus* strains. The factor by which rifampin resistance transformation frequencies were reduced compared with reference transformation frequencies was taken as a measure of what Roberts and Cohan (296) called sexual isolation. Variations of the nucleotide sequence of PCR products from the isolates in relation to the nucleotide sequence of the PCR product from the *B. subtilis* Marburg strain were identified by differences in restriction fragment patterns within the *rpoB* gene. The results revealed a log-linear relationship between the degree of sexual isolation and sequence divergence. Although the results of this study allow prediction of sexual isolation when the extent of sequence divergence is known, Roberts and Cohan (296) doubted whether the same relationship holds true for other transformable species. Differences in mismatch correction activity, presence of restriction modification systems active against DNA taken up, and the need for a recognition nucleotide sequence for DNA uptake all probably contribute to sexual isolation. In bacteria, genes determining these functions can be considered examples of "speciation genes" as defined for higher organisms (60). The data support the expectation that interspecies transfer of genes by transformation would occur preferentially at conserved loci.

Incidence and level of competence in natural isolates. A high variance of transformability and of the level of competence has been observed among natural isolates belonging to species of which one strain is a common transformable laboratory strain. Of 54 clinical isolates of *Acinetobacter calcoaceticus*, 2 were transformable (4%), the frequencies being 0.3 and 1.9% of that of the standard BD4 strain (20). A qualitative test revealed that 14 of 22 (64%) type culture strains of *P. stutzeri* and closely related species were transformable (38). Of 10 clinical isolates of *Helicobacter pylori*, 3 (30%) were naturally competent, with a 100-fold difference in their transformation frequencies (122). A survey conducted with a collection of

clinical isolates of virulent, encapsulated *S. pneumoniae* showed them all to be nontransformable (395). However, of nine isolates, six developed competence when induced by exogenously added competence factor of a highly transformable laboratory strain. Yother et al. (395) proposed different causes of the nontransformability of natural isolates: (i) the presence of the capsule, which may constitute a physical barrier for the excretion and penetration of the produced competence factor; (ii) the presence of a defect in the production or excretion of the competence factor in some strains; and (iii) a defect in the DNA-processing machinery in strains which were nontransformable even with exogenously added competence factor. Of 31 *H. influenzae* clinical isolates, 13 (42%) were transformable, the frequencies ranging from 0.02 to 10% of that of the standard Rd strain (305). In contrast to *S. pneumoniae*, the presence of a capsule did not affect the transformability of the *Haemophilus* strains. Cohan et al. (51) isolated 771 heat-resistant organisms from 27 samples of desert soil. Of these, 54% were *B. subtilis*. One *B. subtilis* strain from each sample was used to test for transformability by its own DNA (chromosomal DNA with linked rifampin and spectinomycin resistance markers). The results showed that competence was a common phenotype, 24 of 27 (89%) isolates being transformable, although the level was low, varying between 0.001 and 0.6% of the transformation frequency of the Marburg 168 strain. The incidence of natural competence among marine isolates was determined recently (92). Of 30 isolates from marine water, diseased fish, or soil, 3 (10%) were transformed at very low frequencies (up to 3.5×10^{-9}) by a plasmid multimer preparation. Of 95 isolates from other marine waters, 15 (16%) were transformable by homologous chromosomal DNA (rifampin resistance). The isolates identified so far belonged to the genera *Vibrio* and *Pseudomonas*. Naturally competent bacteria have also been found in 5 of 14 concentrated marine microbial assemblages (Table 5).

The studies with the natural isolates of various species suggest that natural selection in the environment does not always act in favor of (i) a high fraction of strains from a species in a natural community being transformable and (ii) a high level of competence of the transformable strains. It is proposed that the effective level of transformation (incidence of transformability and competence level) is genetically adjusted to submaximum values specific for each species in its respective habitat. Certain laboratory strains are overshoot mutants. Among these are *B. subtilis* 168 Marburg (246), *Acinetobacter calcoaceticus* BD4 and its nonencapsulated mutants (158), and *Vibrio* strain WJT-1C which was isolated as a high-frequency-of-transformation mutant from a marine isolate (93).

Physiological effects of DNA uptake. Establishment of a DNA donor sequence either by recombination with the recipient chromosome or by reconstitution of a replicon may be followed by the expression of a newly gained gene. Classically, the appearance of the altered phenotypic trait is the experimental evidence of a transformation event. DNA uptake may also serve other purposes, i.e., protection against bacteriophages, nutrient exploitation, regulation of gene expression, and DNA repair (see the section on biological functions of DNA uptake other than gene acquisition, below). Besides the advantageous outcome of a gene transfer event, for instance by an acquired antibiotic resistance or a new degradative capacity, deleterious effects of transformation have also been noted. Following exposure of competent *H. influenzae* cells to DNA of other *Haemophilus* species, up to 55% of a population was shown to be killed, presumably as the result of induction of a defective prophage (5). In other experiments, a high incidence of mutations at loci which were wild type in both parents was found in chromosomal transformants of the cyanobacterium *Anacystis nidulans* (133) and of *S. pneumoniae* (116). There are indications that maintenance of a plasmid puts an additional metabolic load on the cell which decreases its fitness in the absence of selection for plasmid-encoded functions such as antibiotic resistance (29, 44). Likewise, plasmid transformants of *Azotobacter vinelandii* did not grow well under stress caused by iron limitation (99). The reduced fitness was attributed to the inability of plasmid transformants to synthesize an iron-complexing siderophore (100). The transformants were also impaired in their ability to fix dinitrogen and had a considerably reduced size. Thus, such phenotypes may select against transformants in the natural environment, as suggested by growth studies with soil extract (101).

It may be hypothesized that the above-described deleterious transformation events contribute to the selective pressure which genetically tunes the transformability incidence and competence levels among bacteria in their habitat (see the previous section).

Environmental Level

The influence of physicochemical factors (e.g., types and concentration of ions, temperature, pH) on transformation, which has been studied relatively intensively, was discussed in the preceding sections. Studies on biological factors affecting gene transfer by free DNA in bacterial habitats are rather scarce. Microcosm studies imply that transformation frequencies in the environment are occasionally high (Table 5). What are the factors that influence the frequency of transformation in a given habitat? What determines the actual concentration of transforming DNA in a habitat? Is the overall supply of nutrients an important parameter affecting transformation (see the section on competence development under environmental conditions, above), or are positive and negative interactions between microbial cells and populations major factors which govern frequencies and rates of the gene transfer? An experimental approach related to the last question may be to examine transformation in simple mixed-strain systems. In such studies, *P. stutzeri* grew well and was transformed at normal frequency when cocultured (in the presence of homologous transforming DNA) with *Acinetobacter calcoaceticus*. *P. stutzeri* was transformed even at higher frequency in the presence of an unidentified bacterial soil isolate (17). In contrast, several other unidentified soil isolates and *B. subtilis* Marburg did not affect growth but completely eliminated transformation of *P. stutzeri* as a result of excreted DNases. Lastly, *P. stutzeri* did not survive during cocultivation with *Serratia marcescens* and certain soil isolates of unknown taxonomic classification (17). These mixed-strain experiments suggest that the type of interactions may range from positive and neutral for transformation to inhibition of transformation (e.g., by production of extracellular DNases) and from no effect on growth to killing of the recipient (e.g., by toxin production). Selection of microbial habitats of increasing complexity and appropriate organisms for experimentation could be a promising approach for the stepwise analysis of ecological factors influencing transformation in the environment.

DEDUCTIVE EVIDENCE FOR BACTERIAL TRANSFORMATION

Horizontal gene transfer among bacteria plays an important role in the maintenance by recombination of the genetic plasticity of bacterial populations. This contributes to the

TABLE 6. Deductive evidence for recombination in natural populations of bacteria[a]

Species	Population structure[b]	Mosaic genes	Naturally transformable
Neisseria meningitidis	Panmictic (224)	+(30, 89, 334, 399)	+(41)
Neisseria gonorrhoeae	Panmictic (224)	+(333)	+(218)
Neisseria lactamica	ND[c]	+(223)	ND
Neisseria polysaccharеae	ND	+(223)	ND
Haemophilus influenzae	Clonal (224)	+(174)	+(218)
Streptococcus pneumoniae	ND	+(77, 223)	+(177)
Streptococcus oralis	ND	+(50)	ND
Streptococcus sanguis	ND	+(78)	+(19)
Bacillus subtilis	Panmictic (84, 148)	ND	+(246)
Bacillus licheniformis	Panmictic (84)	ND	+(102)
Rhizobium meliloti	Panmictic (224)	ND	+(59)
Rhizobium leguminosarum	Panmictic (329)	ND	ND
Rhizobium etli	Panmictic (330)	ND	ND
Bordetella bronchiseptica	Clonal (247)	ND	+(cited in 247)
Escherichia coli	Clonal (389)	+(83, 213, 229, 316)	–
Salmonella typhimurium	Clonal (224)	+(117, 142)	–
Legionella spp.	Clonal (313)	ND	ND
Pseudomonas syringae	Clonal (224)	ND	ND

[a] References in parentheses.
[b] Determined by multilocus enzyme electrophoresis (312).
[c] ND, not determined.

genetic adaptation to a changing environment and at the same time provides the basis for speciation through sexual isolation. In vitro studies on horizontal gene transfer processes such as conjugation, transduction, and transformation have provided evidence for transfers within bacterial species, between distant taxonomic groups, and even across kingdom borders (65, 131, 225). Presently, deductive evidence for gene exchange among bacteria (and between bacteria and higher organisms) is obtained from comparative analysis of nucleotide sequences, codon usage, and enzyme patterns. The view that genes and chromosomes of bacteria represent mosaics of parts of different species or of parts with different evolutionary history (223, 326) has been documented by analysis of specific genes in several bacterial species, including pathogenic, commensal, and soil organisms (Table 6). Natural transformation as a mechanism for the acquisition of genetic information has been suggested to contribute to the formation of mosaic structures (223). Data from multilocus enzyme electrophoresis suggest that highly sexual (panmictic) populations were frequently those that are naturally transformable (Table 6), such as *N. meningitidis*, *N. gonorrhoeae*, and *R. meliloti* (224). *B. subtilis* (148, 296) and *B. licheniformis* (84) also belong to this group. The transformability of *R. leguminosarum* and *R. etli*, which, according to multilocus enzyme electrophoresis data, also appear to be sexual species (329, 330), has not yet been demonstrated. The data on transformable *H. influenzae* (224) and *Bordetella bronchiseptica* (cited in reference 247) show little evidence for frequent recombination in nature (Table 6). However, physical genetic mapping provided evidence for localized recombination in *H. influenzae*, which would not change enzyme electrophoretic patterns (174, 223). Physical genetic mapping also provided evidence for interspecies gene transfer among transformable bacteria, particularly between different *Neisseria* species (334, 399) and between *S. pneumoniae* and other streptococci (50, 77, 78). Some details have been described above (see the section on habitat of pathogenic bacteria). In conclusion, there is accumulating deductive evidence for genetic exchange among bacteria, even in those with a clonal population structure. In species which are capable of DNA uptake, gene transfer may rely greatly on natural transformation. Plasmids and transposons are probably also transferred by transformation, as is chromosomal DNA (Fig. 2). Genetic exchange between bacteria and higher organisms has probably also occurred, although some of the initially identified instances have been questioned (326).

BIOLOGICAL FUNCTIONS OF DNA UPTAKE OTHER THAN GENE ACQUISITION

What could have been the cause of the evolution and conservation of uptake systems for free DNA in bacteria? Outcrossing, i.e., sex, may be an advantageous strategy to genetically adapt to a changing environment. From Fig. 2 it is evident that exchange of chromosomal genes is limited mostly to closely related species. The potential for sexual isolation even in bacteria that take up DNA of any source has probably evolved because unrestricted gene acquisition could continuously neutralize the specific genetic adaptation of a species to the prevailing conditions of its habitat. Uptake of DNA with essentially no probability to confer a beneficial new trait to the recipient (either because the genetic information is already present or because the DNA cannot be integrated into the chromosome) may nevertheless be advantageous if the DNA is used for other purposes. Four possible functions of DNA uptake besides general gene acquisition are summarized below.

Regulation of Gene Expression

A role for DNA uptake different from gene acquisition is documented by studies of neisseriae (for reviews, see references 233 and 297). Variation in antigenic properties (phase variation), such as variation of pilin production, is an invasive strategy of pathogenic bacteria. Expression of the pilin genes is regulated by either intrachromosomal recombination between a silent pilin gene and the pilin expression locus or integration of the silent gene on entered donor DNA. This produces new combinations of intragenic minicassettes, which lead to different states of assembly of pili, a system somewhat reminiscent of the mammalian immune response system (233, 297). The

finding that phase variation is increased in a culture as autolysis of some cells starts (DNA release) and that this increase can be reduced by the addition of DNase is evidence for the role of transformation in the regulation of the invasive strategy (233, 297).

Protection of Cells against Bacteriophages

Different strains of *S. pneumoniae* have the restriction systems *Dpn*I and *Dpn*II encoded by alternative gene cassettes at the same locus on the chromosome (179). Both systems recognize the same nucleotide sequence. The restriction endonucleases of the *Dpn*I and the *Dpn*II systems act only on the methylated and only on the nonmethylated recognition sequences, respectively (177). Bacteriophages propagated in a strain with *Dpn*I will be restricted in the *Dpn*II strain and vice versa. By transformation with DNA from a *Dpn*I strain, a *Dpn*II strain can change its restriction system to *Dpn*I, because homologous recombination in the flanking regions will facilitate integration of the restriction enzyme gene cassette. It is possible that protection of natural populations of *S. pneumoniae* against epidemic spread of phages can occur by switching of the restriction system. In *S. pneumoniae*, DNA restriction acts on injected duplex phage DNA but does not prevent transformation by chromosomal DNA (180).

Supply with Nutrients

DNA uptake as a means of acquisition of nutrients has been proposed by Stewart and Carlson (344). The processing of bound DNA during uptake in *B. subtilis* and streptococci (and presumably in *Acinetobacter calcoaceticus*) makes degradation products available for metabolism. Likewise, single-stranded material is subjected to nucleolysis if not incorporated in the chromosome as a result of insufficient homology or if not reconstituted to a replicon (i.e., a plasmid or phage). Nucleotides produced during the degradation of heterologous DNA are readily used as precursors for DNA synthesis (72, 243, 363). It appears that in these bacteria a strategy exists which permits the utilization of DNA as a source of (i) nucleotides for replication; (ii) carbon, nitrogen, and phosphorus for general metabolism; and (iii) genetic information if homology suffices for recombinational integration. This aspect may also apply to *Haemophilus* spp., *Neisseria* spp., *P. stutzeri*, and *Azotobacter vinelandii*, except that they preferentially utilize DNA from the same or closely related species. Support for the "food hypothesis" comes from the observation that the last two organisms respond to nutrient limitation with an increased level of competence (202, 203, 267). There are two reports demonstrating DNA binding to cells of nucleic acid-hydrolyzing natural freshwater and marine communities of microorganisms (276, 281). Competition studies suggested that the binding sites for DNA on the cells were different from those for mononucleotides, mononucleosides, and phosphate. It remains to be shown, however, whether DNA uptake occurs or whether cell-bound DNA is hydrolyzed by cell surface-associated nucleases and the degradation products are then transported into the cell by specific carriers. If DNA uptake has evolved as a strategy for the acquisition of the nutrient DNA, which is ubiquitous (Table 2), it does not exclude other functions of the transformation process, such as recombinational repair of DNA lesions.

DNA Repair

Transformational repair is another possible function of DNA uptake; i.e., incoming homologous DNA is used for recombinational repair of DNA lesions present in the chromosome of the recipient suffering genotoxic stress (235). The repair hypothesis predicts that transformation leads to enhanced survival of competent (sexual) cells compared with noncompetent (asexual) cells in a population. Michod et al. (235) tested the repair hypothesis using the *B. subtilis* transformation system. Competent cultures were treated with different doses of UV either before (UV-DNA) or after (DNA-UV) transformation of a chromosomal marker. The data clearly demonstrated that with increasing UV doses in the UV-DNA treatment, transformation frequencies increased and at their maximum were two- to sixfold greater than in nonirradiated cells, but that they decreased in DNA-UV treatments. The same feature was observed in experiments with damaged donor DNA extracted from UV-irradiated cells, a condition more closely resembling natural situations (137). The reason for the increased survival of transformed cells was further investigated. Enhancement of DNA binding or uptake was ruled out since frequencies of plasmid transformation, which proceeds via the same uptake pathway as chromosomal DNA transformation but was used to tag the competence level of the culture because of its independence on *rec* functions, was unaffected by UV treatments (235, 392). Similarly, involvement of excision repair or SOS repair in the enhanced survival of transformed cells seemed improbable since an increase of transformation frequencies in UV-DNA experiments was observed with an excision repair-deficient mutant (*uvrA42*) and the SOS response/DNA repair-impaired *recA1* mutant (which has only a moderately reduced capacity for transformation by chromosomal DNA [392]). Although all these data suggest a benefit of transformational repair to the survival of *B. subtilis* in a genotoxic environment, recent findings with *H. influenzae* are in conflict with the repair hypothesis. A small increase in survival was noted when an *H. influenzae* culture was transformed with chromosomal DNA after UV irradiation. The same effect was observed when the donor DNA consisted of a plasmid containing a chromosomal fragment which forms less than 1% of the genome or when replication of cells was inhibited by oxolinic acid (239). The following explanation was given: DNA integration inhibits replication, thereby extending the time for excision repair to remove lethal damage. Redfield (292) argued that if DNA repair is the primary function of transformation, one would expect competence to be regulated by DNA damage. However, this is not the case in *B. subtilis* and *H. influenzae* (292). On the other hand, RecA and damage-inducible gene products are present at elevated levels in competent cells (see reference 292 and references therein). The failure of regulation by DNA lesions does not, however, exclude transforming DNA as a template for recombinational repair. Redfield (292) further argued that repair was not a strong enough benefit for the evolution of damage-responding regulatory mechanisms of DNA uptake capability. Rather, it is conceivable that recombinational adaptation, protection against phages, DNA repair, acquisition of nutrients, and perhaps regulation of gene expression form a combination of functions with evolutionary potential and led to the conservation of the DNA uptake capability in bacteria.

CONCLUSIONS AND PERSPECTIVES

Recently a large body of evidence has been gathered that suggests that horizontal gene transfer by genetic transformation of bacteria occurs in the environment. The continual production and release of DNA by bacterial populations and the relatively long persistence of this DNA, particularly when associated with solid surfaces, provide extracellular gene pools

in the bacterial habitats despite the ubiquitous presence of DNases. Naturally transformable bacteria in the habitat can take up DNA and propagate its genetic information either when the DNA is present in the dissolved state or when it is associated with particulate material or with cells. Many species with such a potential have been identified among all major taxonomic and trophic groups. In extensive studies, chemical, physical, or biotic parameters that would absolutely preclude transformation in the environment were not found. There is evidence that the flow of genes via free DNA in the environment can be relatively high but is genetically adjusted to a submaximal level. Gene transfer by transformation occurs within species but can also occur between different species and genera and may encompass chromosomal and plasmid DNA. Transformation is thought to play a profound role in the genetic adaptation of bacterial populations to environmental conditions and, as a sexual process, to contribute to evolution and speciation. The genetic analyses of bacterial populations suggest that the observed mosaic structure of genes and genomes is the footprint of gene transfer processes. Among naturally transformable species, genes may pass by genetic transformation.

There are still many questions and a lack of knowledge of important details. The investigation of the physiology and genetics of the process of natural transformation, in the past limited to only a few organisms mainly of medical interest, will extend to more species, including bacteria living in aquatic and terrestrial habitats. The identification of genes and their functions will be the first step and will help to find more naturally transformable species from various environments by use of gene probes. The abundance of transformable species in the natural habitats could be traced. Gene probes may also allow us to systematically screen for transformation genes in bacteria of taxonomic groups from all branches of the prokaryotic phylogenetic tree. Molecular studies can provide further insights into regulatory mechanisms acting on transformability at the cellular level (e.g., control of expression of relevant genes) and at the environmental level (e.g., identification of environmental parameters that are important and how are they sensed by bacteria). Moreover, by characterization of the genes involved, insights into the evolution of natural transformation will be gained. In particular, this can clarify the relation of the transmembrane DNA translocation processes during transformation to other transmembrane transport systems, including those active in conjugative DNA transfer, protein transport, DNA and RNA excretion, and phage infection. Other studies will have specific ecological relevance. The abundance and distribution of DNA in complex environments such as soils and the actual fluctuations in concentration are not yet known. Methods to identify and quantify extracellular (in particular transforming) DNA in the environment will have to be developed. Relevant data on the free DNA pool and its turnover will complement knowledge of the physiology of transformable species and may allow estimation of frequencies and rates of transformation events in bacterial habitats. Such estimates are important for risk assessments associated with releases of genetically engineered organisms into the environment. Other questions may also be asked. What are the determinants of actual transfer rates in situ, and what influences spatial and temporal dynamics of transformation? Is there a coupling of DNA release and transformation to population dynamics? Might there be a correlation between sexuality and reproduction in bacteria? Experiments with microcosms will progressively be paralleled by studies in the environment. The present work with a limited number of exemplary "model" microorganisms will be broadened to include more organisms, possibly from extreme environments. Transformation processes between members of natural bacterial communities will be investigated. Also, the physiological stages that bacteria experience in their habitats, such as starvation and dormancy, have to be considered when production of extracellular DNA and transformation are examined. Despite the recent exciting developments, the research on bacterial gene transfer by transformation in the environment is still at its beginning. It can be seen as an important part of the general endeavor to explore the microbial ecology of the aquatic and terrestrial environments surrounding us. The potentials and dynamics of gene transfer are a vital part of the dynamics of bacterial communities and ecosystems, and genetic transformation probably plays a key role in bacterial gene transfer.

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