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THE ROYAL SOCIETY

Evolution of microbial pathogens

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Various genetic mechanisms including point mutations, genetic rearrangements and lateral gene transfer processes contribute to the evolution of microbes. Long-term processes leading to the development of new species or subspecies are termed macroevolution, and short-term developments, which occur during days or weeks, are considered as microevolution. Both processes, macro- and microevolution need horizontal gene transfer, which is particularly important for the development of pathogenic microorganisms. Plasmids, bacteriophages and so-called pathogenicity islands (PAIs) play a crucial role in the evolution of pathogens. During microevolution, genome variability of pathogenic microbes leads to new phenotypes, which play an important role in the acute development of an infectious disease. Infections due to *Staphylococcus epidermidis*, *Candida albicans* and *Escherichia coli* will be described with special emphasis on processes of microevolution. In contrast, the development of PAIs is a process involved in macroevolution. PAIs are especially important in processes leading to new pathotypes or even species. In this review, particular attention will be given to the fact that the evolution of pathogenic microbes can be considered as a specific example for microbial evolution in general.

Keywords: evolution; pathogenicity; genomic island; biofilm; codon usage; resistance

1. INTRODUCTION: MICROEVOLUTION AND MACROEVOLUTION

The scientific observations on the evolution of organisms made by Charles Darwin (1809–1882) in the 19th century are not only true for eukaryotic organisms, they also seem to be valid for prokaryotes. The key processes of Darwinian evolution can be described by four different termini: genetic variability, phenotype formation, selection and isolation (figure 1). There is no doubt that the permanent development of new genetic variants represents the main requisite for the development of life. As many genetic alterations (mutations) do not lead to new phenotypes it is crucial to point out that only the alterations that do produce new phenotypes are decisive for evolution. New phenotypic variants are immediately subject to selection by biological and non-biological forces. As already described by Darwin, the geographical isolation of certain species or groups of organisms may be useful for evolutionary development. In addition, a genetic isolation due to transfer barriers, strong restriction-modification systems or changes in the codon usage may also contribute to the speed of evolutionary development.

The first micro-organisms appeared on Earth more than three billion years ago. As humans have developed in the last 1.5 million years, strictly human pathogens can be considered as very young microbes. Nevertheless, evolutionary development is also seen in these microbes. An evolutionary process that occurs within a longer period of time and that leads to the formation of new species or subspecies is considered as macroevolution. According to Ernst Mayr, macroevolution is the key developmental process for the evolution of life.

In contrast, processes of microevolution take days or weeks. As a consequence of microevolution, new variants of a certain species or subspecies are generated. During evolution, 'variability generators' such as insertion sequence (IS) elements, switching DNA fragments or transposons have appeared, which play a key role in microevolution. Microevolution is extremely important for the pathogenesis of infectious diseases. The expression or non-expression of particular genes (phase variation) or the alteration of microbial structures, especially surface structures such as pili or outer membrane proteins (antigenic variation) *in vivo* represent paradigms of microevolution. In addition, these processes are virulence mechanisms, important in many infectious diseases.

In this article we will first describe the general genetic mechanisms involved in the development of microorganisms. Particular attention, however, will be given to pathogenic microbes. It is our view that pathogens produce many virulence or pathogenicity factors, which directly or indirectly contribute to the development of an infectious disease. These factors and the underlying gene clusters are of particular importance for the evolution of pathogens. In addition, pathogenic micro-organisms express resistance factors, which render the organisms resistant to antimicrobial drugs. The genetic basis of antimicrobial drug resistance is also an example for 'evolution under the microscope'. In this review we will present examples for microevolution of virulence factors as well as resistance mechanisms; both are important in various

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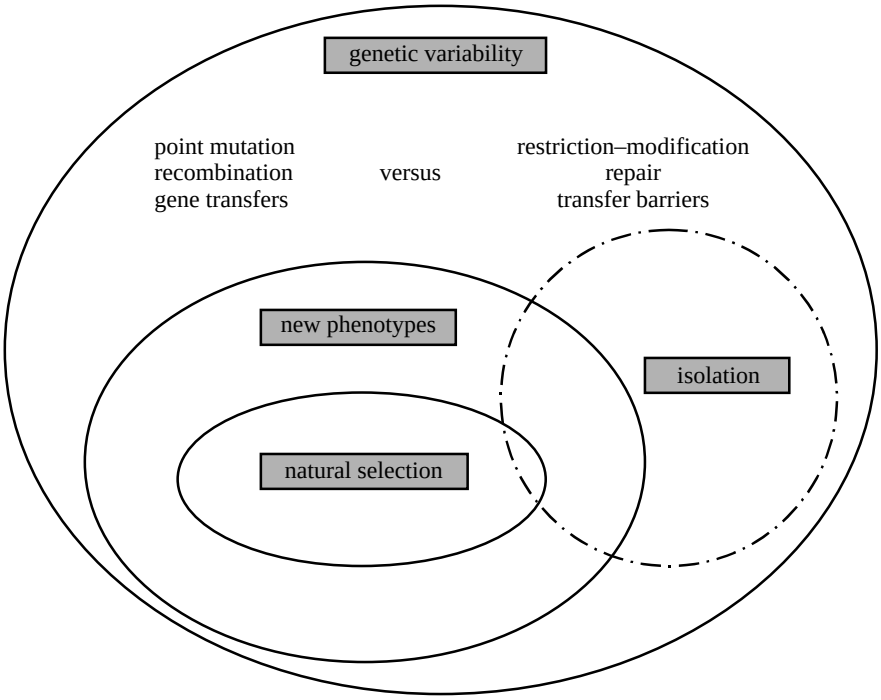

Figure 1. Darwinian principles of evolution.

microbe host systems. Finally, the present knowledge on processes of macroevolution of pathogens with special emphasis on the occurrence and structure of pathogenicity islands (PAIs) will be summarized.

2. MECHANISMS OF GENETIC VARIABILITY

The evolution of organisms relies on heritable changes that are transmitted to the offspring. Phenotypic alterations that confer a selective advantage therefore must have a genetic basis to be relevant in evolutionary terms. There are several different mechanisms by which microorganisms produce genetic variability: the accumulation of point mutations, genetic rearrangements and the acquisition of new genetic material by horizontal gene transfer.

(a) *Point mutations*

Point mutations occur more or less randomly throughout the genome and are generated by replication errors or incorrect repair following DNA damage. They can be silent; for example, when they are introduced into the coding sequence of a gene without altering the amino-acid sequence of the gene product. However, if the mutation results in an amino-acid exchange, a modified protein will be the consequence. Alternatively, such mutations may also occur in regulatory regions, thereby affecting the expression pattern of the respective gene(s). Single point mutations usually affect one specific trait that may confer an advantage in a changing environment (Musser 1995). This can generate new variants of a clone within relatively short periods of time (microevolution). On a larger scale, i.e. the generation of new species, evolution by accumulation of point mutations is a very slow process. This is especially true for mutations in essential species-specific housekeeping genes, e.g. loci encoding ribosomal RNAs, particular enzymes (for example ATPases) or structural proteins.

(b) *Genetic rearrangements*

Microbes may alter their genome also by rearrangement of existing parts. For example, gene amplifications result in an increase in the number of blueprints available for production of the corresponding gene product, which would be of advantage in an environment that demands a constantly higher expression. Such amplifications may be caused by recombination between repetitive DNA elements flanking the amplified sequence. Gene duplications also provide the material for further mutational evolution of the redundant copies without destroying the function of the product encoded by the original gene. In this way, variants can be generated with fine-tuned functional and/or regulatory properties. Recombination between individual members of gene families generated by duplications provides an additional level of variability, leading to new variants with altered properties by shuffling modules from pre-existing copies. The same genetic rearrangements that cause amplifications can also result in the loss of parts of the genome that are not essential any longer when a microbe has adapted to a new ecological niche, thereby freeing the organism of this genetic burden. Mobile genetic elements like IS elements are frequently substrates of such recombination events; however, they can also generate variability by their insertion into new sites in the genome, which may activate or inactivate genes located at those sites.

(c) *Gene transfer*

Microbes may alter their characteristics much more rapidly by acquisition of new genetic material from other organisms. Three basic mechanisms of gene transfer are known: transformation, transduction and conjugation (the mating between bacteria). Some bacteria, like *Neisseria gonorrhoeae* or *Haemophilus influenzae*, have a high natural competence to take up free DNA from lysed cells of their own species, which may subsequently recombine with corresponding genomic sequences within the cell to

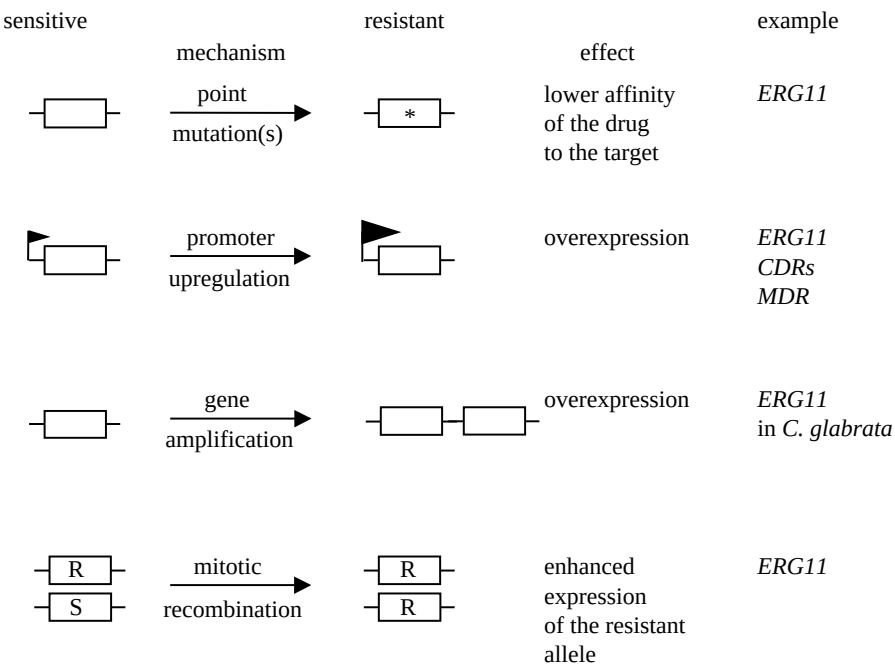

Figure 2. Molecular mechanisms of azole resistance in *Candida*. The schematic representation shows the genetic mechanisms which lead to phenotypes with reduced susceptibility. Examples of the genes involved in azole resistance are indicated (see § 3(a)).

generate new variants. Within a given species there is often a great variety of individual organisms, especially in pathogenic bacteria that may or may not possess genes encoding virulence factors or antibiotic resistance determinants. Such genes may be located on the chromosome, on transposable elements, conjugative plamids or on phages that have integrated these genetic elements into their genome, and can be transferred to members of the same species or even to other species by conjugation or by phage transduction. These mechanisms are very important for the evolution of pathogenic bacteria since, instead of the slow adaptation of their own molecules to new functions, they allow the rapid generation of new variants with considerably altered properties by the exploitation of 'ready to use' genetic material from other organisms.

3. EXAMPLES FOR MICROEVOLUTION OF PATHOGENS

(a) *Microevolution in* Candida albicans

The opportunistic fungal pathogen *C. albicans* is a harmless commensal in many healthy humans, residing on the mucosal surfaces of the gastrointestinal and urogenital tract. In immunocompromised patients, however, *C. albicans* can cause superficial as well as life-threatening disseminated infections. AIDS patients with oropharyngeal candidiasis or women with *Candida* vaginitis frequently suffer from recurrent infections, which are in most cases caused by the same *C. albicans* strain (Lischewski *et al*. 1995). Sensitive fingerprinting methods have shown that these strains undergo microevolution, as demonstrated by subtle changes in the DNA fingerprint pattern between individual isolates from different infection episodes (Lockhart *et al*. 1996; Schröppel *et al*. 1994).

Oropharyngeal candidiasis in AIDS patients is usually treated effectively with azole antifungals, especially fluconazole, which inhibit the biosynthesis of ergosterol, the major sterol in the fungal plasma membrane. Since the introduction of fluconazole and its broad use during the past decade, resistant *C. albicans* strains have emerged in patients receiving long-term fluconazole treatment, resulting in therapy failure (White *et al*. 1998). As compared with previous isolates from earlier episodes, the resistant isolates have frequently undergone several genomic changes that contribute to their reduced drug susceptibility (figure 2). For example, point mutations within the *ERG11* gene, encoding the drug target enzyme sterol 14α-demethylase, result in the production of an enzyme with lowered affinity for the drug (Franz *et al*. 1998; Sanglard *et al*. 1998; White 1997*b*). Additionally, chromosomal rearrangements may contribute to enhanced resistance, because mitotic recombination in the diploid *C. albicans* can lead to homozygosity for a mutated *ERG11* allele (Franz *et al*. 1998; White 1997*b*), such that the cell produces only the resistant version of the enzyme. Similarly, constitutive overexpression of the target gene has also been observed, and this in turn may result in enhanced enzyme activity within the cell (Franz *et al*. 1998; White 1997*a*). In *C. albicans*, *ERG11* overexpression is probably caused by enhanced promoter activity, whereas in *C. glabrata* gene amplification has also been demonstrated (Marichal *et al*. 1997).

In addition, *C. albicans* possesses efflux pumps that enable the fungus to transport drugs actively out of the cell. In many fluconazole-resistant isolates a reduced intracellular accumulation of the drug correlating with stable, constitutive overexpression of these membrane transport proteins was observed (Sanglard *et al*. 1995, 1997). The ATP-binding cassette transporters Cdrlp and Cdr2p transport other azoles in addition to fluconazole. Consequently, *CDR1* and/or *CDR2* overexpression confers cross-resistance to several different azoles. In contrast, the substrate specificity of the Mdrl protein, which belongs to the major facilitator superfamily, is more limited. *C. albicans* strains in which fluconazole resistance correlates with activation of the *MDR1* gene are still

susceptible to ketoconazole and itraconazole, although several other unrelated drugs are transported by this efflux pump (Goldway *et al*. 1995; Sanglard *et al*. 1995, 1996). The exact nature of the mutations leading to overexpression of membrane transport proteins has not yet been clarified, but it seems that, similar to the baker's yeast *Saccharomyces cerevisiae*, mutations in regulatory proteins controlling the expression of the transporter genes are involved (Carvajal *et al*. 1997; Nourani *et al*. 1997; Wirsching *et al*. 2000).

The genomic alterations described are probably rare events, but once they have occurred the selective pressure exerted by the presence of the drug favours the overgrowth of *C. albicans* cells with increased resistance in a previously susceptible population. The constant microevolution of *C. albicans* results in the generation of strains in which multiple mechanisms have contributed to produce highly resistant strains. Under normal circumstances, the mutations would presumably reduce the fitness of the strains. For example, it has been shown that a mutation in sterol 14α-demethylase lowering the affinity of the enzyme to fluconazole also reduces enzyme activity (Kelly *et al*. 1999) and therefore might affect the growth rate of the cells when ergosterol biosynthesis is a limiting factor. Similarly, unregulated expression of biosynthesis enzymes or efflux pumps might be unfavourable for variant cells in many host niches as compared with cells that maintained the ability to fine-tune gene expression in response to environmental signals. The selective conditions in the clinical situation, however, give an advantage to cells with mutations conferring drug resistance Therefore, it can be concluded that genomic alterations, especially point mutations and rearrangements, which occur during the *in vivo* process of infection result in microevolutionary development of *C. albicans*.

(b) *Variation of biofilm formation in* Staphylococcus epidermidis *and* S. aureus

In aquatic, nutrient-limited ecosystems many bacteria develop a marked tendency to attach to surfaces and to initiate their organization in biofilms (figure 3). Bacterial biofilms are characterized by the production of slimy matrix substances that enclose the bacterial cells and mediate their adherence to each other and to solid surfaces (Costerton *et al*. 1995). Bacterial biofilms are widespread in nature and, in a sense, their organization can be considered to resemble that of multicellular organisms (Shapiro 1998). This point of view is supported by the fact that bacterial cells in biofilms differ considerably from their planktonic counterparts in terms of metabolic activity, gene expression and an inherent higher resistance to antibiotics.

Numerous pathogens, which are common sources of persistent and recurrent infections, e.g. *Pseudomonas aeruginosa*, *Escherichia coli*, streptococci and staphylococci, have been shown to generate biofilms (Costerton *et al*. 1999). In the course of an infection caused by biofilm-forming bacteria, planktonic cells are constantly released from the sessile population and they obviously are not efficiently eliminated by host defence mechanisms. In this respect, it has been hypothesized that specific detachment programmes exist that, once activated, abolish the extracellular matrix substance expression (Costerton *et al*. 1995). However, the genetic mechanisms of bacterial biofilm detachment are poorly understood. Recently, the molecular basis of biofilm formation in *S. epidermidis* and *S. aureus* has been elucidated and it has been demonstrated that biofilm expression undergoes strong phenotypic and genetic variations, which are believed to contribute to the detachment programmes mentioned above. Phase variation in biofilm formation indeed represents a good example for microevolution of staphylococcal isolates.

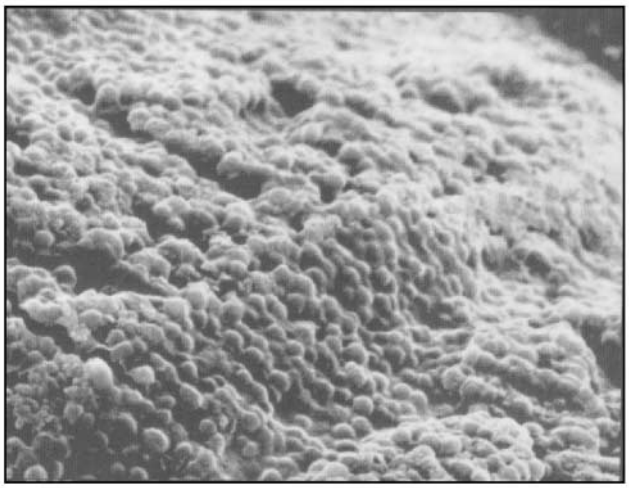

Figure 3. Biofilm formation of an *S. epidermidis* strain on a polystyrene surface.

S. aureus and the coagulase-negative species *S. epidermidis* are the most common causes of nosocomial infections. Most of these infections are associated with indwelling medical devices. The formation of staphylococcal biofilms on plastic material was shown to be mediated by the *ica*ADBC-operon (Heilmann *et al*. 1996). Upon activation of this operon, a polysaccharide intercellular adhesin (PIA) is synthesized that mediates biofilm production. The evolutionary origin of the *ica* genes is not known. It appears, however, that this genetic information is widespread in clinical *S. epidermidis* isolates (Ziebuhr *et al*. 1997). In contrast, it is rarely observed in saprophytic strains from the healthy mucosa. With regard to *S. aureus* it has been found that all isolates, regardless of their origin, contain the *ica* genes (Cramton *et al*. 1999). However, only very few strains indeed express the operon and, consequently, most *S. aureus* strains are biofilm negative *in vitro*. Apart from these genetic differences, the PIA synthesis has been reported to undergo a phase variation process in biofilm-producing strains which is, in a substantial number of variants, caused by the alternating insertion and excision of the mobile genetic element IS*256* into (from) different sites of the *ica* gene cluster (Ziebuhr *et al*. 1999*a*) (figure 4). Especially, the *ica*C gene seems to represent a preferred target for IS*256* insertions. This study also revealed the reversible nature of this transposition. Thus, following repeated passages of PIA-negative insertional mutants, the biofilm-forming phenotype could be restored. Nucleotide sequence analyses of the revertants confirmed the complete excision of IS*256*. Recently, *ica*C::IS*256* insertional mutants were detected in clinical isolates from patients with indwelling medical devices and therefore it is assumed that the process might play a significant role during an infection. It is

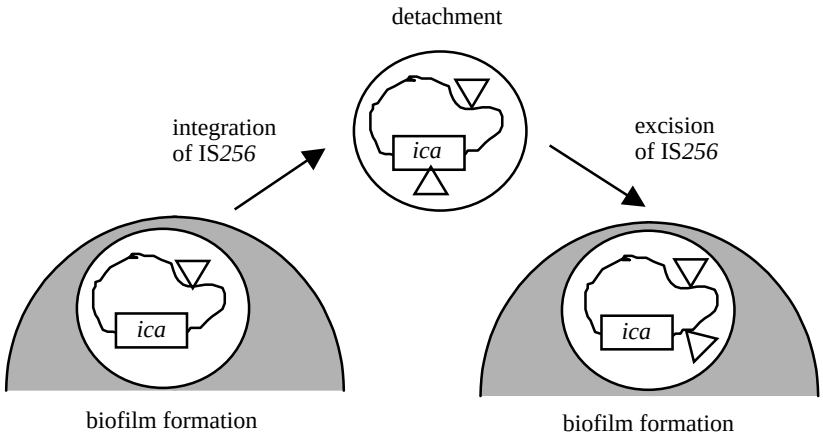

Figure 4. Phase variation in staphylococci. Biofilm-forming bacteria are detached after insertional inactivation of the *ica* genes by IS*256*. Excision of IS*256* enables the cells to recolonize and form another biofilm.

conceivable that the switch-off of the PIA production enables single bacterial cells to detach from the biofilm and to disseminate into novel habitats. A possible back-switch to the biofilm-producing phenotype, at a later stage of infection, would again render them capable of forming new biofilms on suitable surfaces (figure 4). In addition to phase variation, biofilm formation can also be irreversibly affected by the complete loss of the *ica* gene cluster or large chromosomal rearrangements (W. Ziebuhr *et al.* 2000). Chromosomal *ica* deletions were detected in different clinical isolates and comprised DNA fragments of *ca.* 70–100 kb in size. Additionally, chromosomal rearrangements were demonstrated during infections by biofilm-forming *S. epidermidis* strains.

Interestingly, both deletions and rearrangements resulted in altered IS*256*-specific hybridization patterns. The data suggest that the genetic and phenotypic flexibility of this pathogen contributes to its successful adaptation to changing environmental conditions and therefore, might be involved in the persistence and also the relapse of an infection. However, more experimental work is needed to investigate the exact molecular mechanisms of these processes.

(c) *Genomic deletions in pathogenic* E. coli

Some strains of *E. coli* are able to cause intestinal as well as extra-intestinal infectious diseases. Urinary tract infections represent the main diseases due to extra-intestinal *E. coli*. Uropathogenic *E. coli* produce a number of virulence factors such as adherence factors (P-fimbriae, S-fimbriae), toxins (haemolysin, cytotoxic necrotizing factor (CNF) I), capsules and particular iron uptake systems (aerobactin, yersinabactin). Recent studies revealed that uropathogenic *E. coli* producing α-haemolysin on blood agar plates undergo a switch from a haemolytic to a non-haemolytic phenotype. This was independently shown for different uropathogenic isolates such as strain 536 (O6:K15) and strain J96 (O4:K6). The modulation of virulence properties was also demonstrated *in vivo* in a rat pyelonephritis model. As the genetic rearrangements of uropathogenic *E. coli* occur with relatively high frequency during days and weeks, they can be considered microevolutionary processes.

Southern hybridization experiments and other molecular methods indicated that the non-haemolytic phenotype was due to deletion of large fragments of DNA, a mechanism also detected in *S. epidermidis* (see § 3(b)). The deletion in *E. coli* comprises DNA fragments of 70 kb up to 190 kb (figure 5). Genes encoding α-haemolysin and gene clusters responsible for P-fimbriae and CNF are located on this unstable region in the genome of uropathogenic *E. coli* strains. The deletions are *rec*A independent and occur with frequencies of 10^{-4} to 10^{-5}. It was shown recently that they represent site-specific events. The analysis of 40 independently generated deletion mutants of strain 536 showed a unique pattern, which argues for the action of a site-specific integrase–exicase, which may direct this process of virulence modulation. Short direct repeats are involved in the generation of the deletions in both uropathogenic *E. coli* strains J96 and 536. In strain 536 the direct repeats are 16 and 18 bp long. Following the deletion process one copy of the repeated DNA retained in the genome of the deletion mutant (Blum *et al.* 1994). We have indications that deletion formation does not only occur *in vitro* or in animal *in vivo* tests, but also during episodes of chronic urinary tract infections (M. Maibaum, G. Blum-Oehler and J. Hacker, unpublished data). The advantage to generate deletion mutants by microevolutionary processes may be that less pathogenic variants may have a better chance to survive in a late stage of urinary tract infection compared with fully virulent bacteria.

4. MECHANISMS OF LONG-TERM EVOLUTIONARY DEVELOPMENT

(a) *On pathogenicity and genomic islands*

In principle, PAIs belong to the repertoire of virulence gene carriers such as plasmids and bacteriophages. These three genetic elements strongly contribute to the macroevolution of pathogens. Plasmids and bacteriophages as well as PAIs, however, may also be involved in processes of microevolution (Finlay & Falkow 1989; Karaolis *et al.* 1999; Ratti *et al.* 1997; Waldor & Mekalanos 1996). The microevolutionary processes of deletion formation in uropathogenic *E. coli* (see § 3(c)) gave excellent indications of macroevolutionary developments: the formation of PAIs. It was demonstrated that the deleted DNA fragments from strain 536 fulfil the criteria of PAIs. PAIs represent large fragments of genomic DNA which are present in pathogenic microbes but absent in less pathogenic or apathogenic strains of the same species or related species. PAIs often carry more than one virulence gene and additional mobility genes such as integrases and

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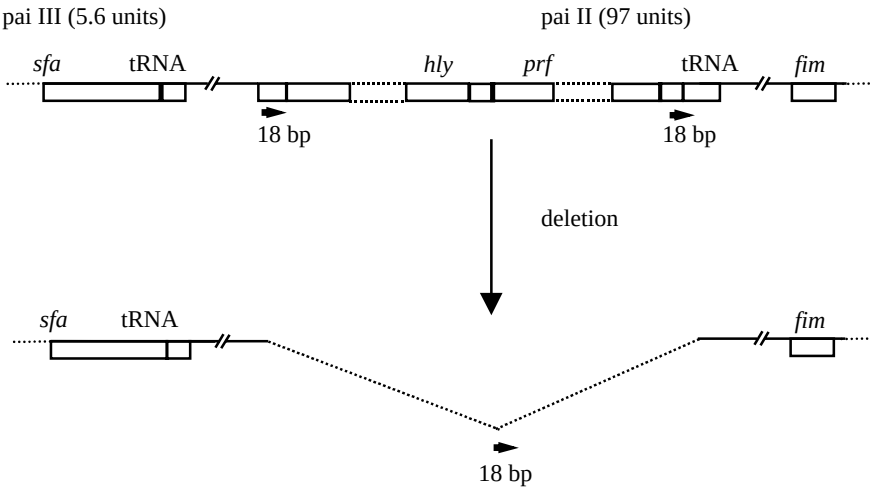

Figure 5. Deletion of a PAI. Spontaneous deletion of PAI II in uropathogenic *E. coli* can occur by recombination between the flanking 18 bp direct repeats. Figure not drawn to scale.

parts of IS elements. PAIs represent distinct sections of DNA with repeats or IS elements at their boundary and they are frequently associated with tRNA genes. In addition, PAIs are highly unstable. They may represent former transferable elements, which have been fixed in the genome of certain species and subspecies during evolution (Dobrindt & Hacker 2000; Hacker *et al.* 1997; Hacker & Kaper 1999).

Strain 536 carries characteristic PAIs with genes encoding P-fimbriae and α-haemolysin. The PAIs are associated with tRNA genes: PAI I is accompanied by the selenocysteine-specific tRNA gene *sel*C, PAI II is located in the vicinity of the tRNA gene *leu*X, which encodes for the minor $tRNA_5^{Leu}$. One should mention here that the *leu*X-specific tRNA seems to act as a specific modulator of gene expression in pathogenic *E. coli*. The absence of the *leu*X-specific tRNA following deletion of PAI II leads to a decrease in expression of a number of virulence factors such as flagella, haemolysin or type I fimbriae (Dobrindt *et al.* 1998; Ritter *et al.* 1995, 1997). Additionally, the $tRNA_5^{Leu}$-specific gene, *leu*X, is part of a regulatory network, because it is regulated by certain global response regulators such as alternative σ-factors (Dobrindt & Hacker 2000). This example illustrates the complex genetic interplay between the core chromosome, PAIs and particular regulators involved in expression of virulence-associated genes.

PAIs seem to be established during long-term processes, which are macroevolutionary. It was shown that certain PAIs specific for a pathogenic *E. coli* strain such as the yersiniabactin-specific island or the locus for enterocyte effacement element of enteropathogenic *E. coli* have been part of the *E. coli* genome for more than one million years. The *Salmonella enterica* genome carries at least five different *Salmonella* PAIs (SPI). SPI I seems to have been present in the genome for more than 100 million years, because it is not only present in the genome of *Salmonella enterica*, but also in the genome of *Salmonella bongeri*. Both species, however, diverged about 100 million years ago. Therefore, it seems that PAIs represent former mobile DNA elements, which became fixed in the genome of certain pathogenic bacteria. If a pathogenic variant is successful, evolutionary pressure leads to the 'homing' of the island in the genome. This may promote the establishment of new pathotypes such as uropathogenic or enteropathogenic *E. coli* or certain serotypes of *Salmonella enterica*.

The increasing number of fully sequenced bacterial genomes reveals that PAIs do not represent unique DNA elements specific for pathogenic bacteria (Buchrieser *et al.* 1998; Censini *et al.* 1996; Lindsay *et al.* 1998). Similar DNA elements though encoding different functions were also found in non-pathogenic bacteria (table 1). The *mec*A gene responsible for methicillin- and oxacillin-resistance encodes an alternative penicillin-binding protein of pathogenic staphylococci. It is also located on a large and unstable DNA fragment that fulfils the criteria for a PAI with the only difference that the fragment harbours a resistance determinant instead of virulence genes. This element could therefore be termed a 'resistance island' (Ito *et al.* 1999). Following integration, a large 500 kb plasmid, which carries the *nif* genes specific for nitrogen fixation in particular strains of *Mesorhizobium loti* was designated as a 'symbiosis island' (Sullivan & Ronson 1998). In addition, conjugative transposons of *Salmonella senftenberg* were denominated as 'metabolic islands', because they carry genes involved in sugar uptake (Hochhut *et al.* 1997). Various bacteria harbour genes for type III secretion (Bonas 1994) or iron uptake on plasmids or distinct sections of the genome. In order to bring all these different structures into a category we propose to use the term 'genomic islands' for such additional elements of DNA with the capacity to encode particular functions of microbes. These genomic islands are not present in all isolates of a species, are unstable, represent a particular fragment of DNA and are very often associated with tRNA genes. It is our opinion that PAIs represent a subgroup of genomic islands. Genomic islands are not restricted to pathogens, rather they are present in the whole microbial world. Figure 6 depicts the possible contributions of the various genetic elements to the core genome of prokaryotes.

(b) *The role of codon usage*

As already mentioned, isolation of organisms or genetic barriers, which may lead to a separation of the genetic material between different groups of organisms, may also play an important role in the evolution of species. One example of 'genetic isolation' is the establishment of an alternative codon-usage programme as found in some organisms. After the discovery that the genetic code is universal in all organisms from bacteria to man it was a

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Table 1. *Examples of genomic islands*

organism	property	type of island	genetic feature
Mesorhizobium loti	N_2 fixation	symbiosis	500 kb plasmid
S. aureus	MecA protein	resistance	51 kb element
Pseudomonas putida	phenol degradation	degradation	104 kb plasmid
Salmonella senftenberg	sucrose uptake	metabolism	45 kb conjugative transposon
various bacteria	type III secretion	secretion	part of chromosome or plasmid
various bacteria	iron uptake	fitness	part of chromosome or plasmid

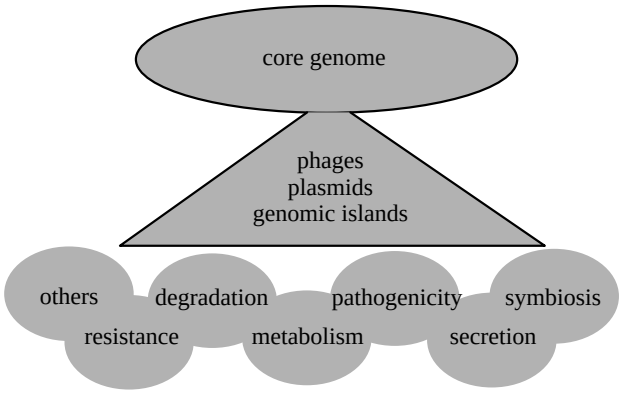

Figure 6. Genome structure of prokaryotes. Various genetic elements like genomic islands, plasmids and phages can integrate in the core genome and deliver new traits to the micro-organism.

long-held view that codon changes would not be allowed during evolution because, by affecting so many genes, this would be a lethal event for the cells (Crick 1968). However, many exceptions concerning specific codons have since been discovered in bacteria, mitochondria and lower eukaryotes (Dybvig & Voelker 1996; Osawa *et al*. 1992). There are two theories of how such alterations in the genetic code might have occurred. The 'codon capture theory' implies that a codon first disappears from the genome due to a drift to extremely high or low GC contents and reappears later with a different meaning. In contrast, the 'ambiguous intermediate theory' postulates that a codon can be recognized by its cognate as well as a near-cognate tRNA and thus be translated into two different amino acids within the same cell before it is eventually taken over by the new tRNA. This implies that codon ambiguity would not be lethal.

Most *Candida* species including the pathogenic species *C. albicans* translate the normally leucine-specific codon CUG to serine, which is the only known example in which a sense codon in a cytoplasmic mRNA has been reassigned (Santos *et al*. 1997; Sugita & Nakase 1999). The ser-$tRNA_{CAG}$ which translates the leucine CUG codon as serine has an unusual structure which lowers its decoding efficiency, thus allowing cells to survive low-level serine CUG translation (Santos *et al*. 1996). At least in some *Candida* species the CUG codon is still ambiguous, i.e. it is read both as serine and as leucine. It has been shown that the codon ambiguity generates a general stress response, which allows cells to grow under normally lethal conditions. This could have been the selective force that provided an advantage of codon ambiguity under stress conditions and enabled *Candida* species to adapt to new ecological niches. The constitutive tolerance of *C. albicans* to stress conditions like high temperature and oxidants might have evolved as a consequence of CUG reassignment, which could therefore also have played a role in the evolution of *Candida* pathogenicity (Santos *et al*. 1999).

(c) *Bacterial IS elements as 'variation generators'*

Bacterial IS elements are small mobile DNA units that encode only features necessary for their own mobilization. In general, they consist of the genetic information for a transposase protein and inverted repeat sequences that exactly define the borders of the element. IS elements can be regarded as repetitive DNA sequences that are randomly distributed on the bacterial chromosome. They also occur on plasmids, phages and in composite transposons where ISs often form the ends of the element. IS elements are known to play an important role in the microevolution of the bacterial genome (Arber 1993; Mahillon & Chandler 1998; Ziebuhr *et al*. 1999*b*), their 'genetic programme', however, has been developed over millions of years. Therefore, the existence of such 'variation generators' (Arber 1993) is a matter of macro-evolutionary processes.

IS elements are also involved in the variation of gene expression during infection. Thus, IS elements have the capacity to cause irreversible inactivation of genes by random and in some cases also by site-specific transposition. Additionally, in *Neisseria meningitidis* and *S. epidermidis* it was shown that distinct elements (i.e. IS*1301* and IS*256*, respectively) can also contribute to reversible inactivation of virulence genes. The *ica* genes of *S. epidermidis* (see § 3(b)) encoding the production of the polysaccharide adhesin PIA represent the target sites for integration and precise excision of IS*256*, leading to repression and reactivation of the *ica*-specific genes (Ziebuhr *et al*. 1999*a*). In *N. meningitidis*, the IS*1301* element mediates phase variation of capsule synthesis by a similar mechanism (Hammerschmidt *et al*. 1996). Both the findings in *Neisseria* and in *S. epidermidis* suggest a general role of bacterial IS elements in the modulation of gene expression in pathogenic bacteria.

Another interesting property of IS elements is their capacity to control the expression of adjacent genes. Numerous elements were shown to contain outwardly directed −35 promoter boxes in their terminal inverted repeats. When such an element transposes at the correct distance from a resident −10 promoter hexamer, new promoters capable of activating genes located downstream are created. These effects have been shown, for

example, for the expression of the aminoglycoside resistance genes in Tn*4001* or for the expression of methicillin-resistance-associated genes in *S. aureus* (Maki & Murakami 1997; Rouch *et al*. 1987).

In addition to simple transposition, IS elements also give rise to complex DNA rearrangements including deletions, inversions, gene amplifications and the fusion of two DNA molecules by co-integrate formation (Arber 1993). In addition, the acquisition of genetic material or its distribution to other strains or species is often mediated by IS elements. Recent studies in enterococci indicate that different IS elements contribute to the evolution of large composite transposons that encode antibiotic resistance genes and carry integrated copies of plasmids (Bonafede *et al*. 1997; Rice & Carias 1998). The harboured genes are then mobilized to susceptible strains by horizontal gene transfer.

In general, the effects described above are mediated by specific actions of IS transposases. However, IS elements are also passively involved in recombination, since their nucleotide sequences represent homologous DNA stretches which might serve as recombinational cross-over points. Apparently, these IS-mediated mechanisms play a major role in genome flexibility. They are involved in the creation of new antigenic variants and since many IS elements are associated with antibiotic resistance genes, they also contribute considerably to the spread of resistance among bacterial populations.

(d) *Gene expression: the right gene at the right time*

All organisms must have the capacity to respond to changing environmental conditions by adapting the expression pattern of their genes. In pathogenic microbes, there are two fundamentally different important principles of changing the expression of genes: the regulated activation and repression of genes in response to external stimuli, and the transition between expressed and non-expressed states, which is usually random and results in phase or antigenic variation (Deitsch *et al*. 1997; Miller *et al*. 1989). The latter is often observed with structures on the cell surface that interact with host cells, like capsules or adhesins (Ölschläger *et al*. 1997). In certain situations, expression of these structures may be advantageous for the cell, whereas in others it may also represent a handicap. For example, the presence of a capsule renders *N. meningitidis* resistant to host defence mechanisms, but it hinders invasion into epithelial cells. Similarly, adhesins are necessary for tissue colonization, but they delay the crossing of the bacteria through mucus and can also mediate binding to phagocytic cells that destroy the bacteria. In addition, surface structures are often immunogenic, and the expression of variants with altered antigenic properties helps the cells to escape from the immune system. Micro-organisms have therefore evolved mechanisms that allow them to switch the expression state of such structures, thereby generating variants from a homogeneous population that gain access to new host niches or evade the host's immune response (Pouttu *et al*. 1999; Sokurenko *et al*. 1999). The genetic loci involved are often hypermutable, which allows the bacteria to rapidly produce the necessary diversity from a clonal population of infecting cells (Moxon & Tang, this issue).

As discussed above, gene families generated by duplication of an ancestral gene allow the evolution of variants which may be better adapted to specialized functions or environmental niches. Correspondingly, regulatory mechanisms have also evolved that allow for the expression of the proper variant under different environmental conditions, which is induced by external signals. *C. albicans* secretes aspartic proteinases that seem to be involved in the pathogenicity of the fungus. The role of this virulence factor during infection has not yet been clarified, but it may include the degradation of tissue barriers, the evasion of host defence mechanisms, or simply the supply of nitrogen sources. *C. albicans* possesses a family of at least ten homologous genes encoding secreted aspartic proteinases, and the individual members of this gene family are differentially regulated *in vitro* (Hube *et al*. 1994). Such a differential regulation is also ensured by host signals during infection, suggesting that the various proteinase isoenzymes might each have a specialized function in different host niches and at different stages of the infection (Staib *et al*. 1999).

5. CONCLUSIONS

In this review we intended to demonstrate that the general mechanisms of Darwinian evolution, generation of genetic variability, phenotypic expression of new variants and selection as well as physical and genetic 'isolation' are also valid for the evolution of microbial pathogens. Therefore, the development of pathogens can be considered as an example of Darwinian evolution. The generation of new variants of microbes also leads to the generation of new pathogens, which may represent new pathotypes belonging to the same species or forming new pathogenic species of microbes. The formation of PAIs is paradigmatic for the generation of new pathogenic variants. In addition, isolation of certain groups of organisms is also a key process of Darwinian evolution. The change of codon usage in pathogenic microbes such as *C. albicans* or *Mycoplasma* spp. illustrates these processes of genetic isolation. Isolated variants have the capacity of rapid development in particular ecological niches.

The mechanisms of microbial evolution elucidated in pathogenic bacteria might not be limited to this group of micro-organisms. While recent data illustrate the evolution of pathogenic bacteria, many processes inherent in the evolution of eukaryotic pathogens are still undiscovered. It will be one major future goal in molecular pathogenesis research to study evolution of pathogenic protozoan and fungal organisms to get further insights into the evolution of eukaryotes.

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