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Review article.

Different Lifestyles of Human Pathogenic Procaryotes and Their Strategies for Phase and Antigenic Variation

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Abstract

Bacteria generally cause disease in a mammalian host by a multifacetted process involving numerous components, each one of which may be necessary for pathogenesis. These virulence determinants include a variety of factors that enable colonization of a specific niche in the host, evasion of host immune responses, invasion of mammalian tissues and/or cells, or direct host toxicity. The mechanisms used by pathogenic bacteria to cause infection and disease are diverse, but they usually include a co-regulated group of complementary genetic properties that are best suited for the interaction of a particular microorganism with a particular host. However, because of their need to overcome similar host and environmental barriers, common themes of microbial pathogenesis have evolved in quite distinct bacterial species. We describe here four different but rather general strategies of host-pathogen interactions and outline the crosstalks which take place at the molecular level during different stages of an infection. Because they deal with various microenvironments during the course of infection, most bacterial pathogens evolved different mechanisms for generating genetic variation. The ability to vary their cell-surface composition may often play a key role in the evasion of antigen-specific host immune defences. In addition, it may promote expression of the most appropriate cell-surface structures for a given microenvironment or infection stage, thereby optimizing the virulence potential of a bacterial pathogen during the course of infection. Here we review, in the context of the corresponding infection processes, mechanisms that bring about this antigenic and phase variation as well as its functional consequences.

Keywords: Host-pathogen interaction, bacterial infection, virulence factors, toxin, epithelial cell invasion, immune escape, genetic variation, pilus, LPS

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1. Different Pathogenic Lifestyles

Classification of pathogenic bacteria according to their lifestyles within the host can never be complete and strict because of the versatility of host-pathogen crosstalks and the fact that virulent bacteria may combine different infection strategies in order to successfully infect the host. Even so, Fig. 1 shows an attempt to group bacterial infection strategies into four general families which are recurrently found when bacteria are causing disease. A few paradigms of bacterium-host interactions are presented for every group. These paradigms were selected with respect to their capabilities of phase and antigenic variation during the course of infection.

A. Plasmin-dependent invasion and dissemination (Fig. 1A)

A key feature of certain pathogenic Gram-positive and Gram-negative bacteria is the ability to invade from cutaneous and mucosal surfaces to deep tissue site. This is further facilitated if mechanical or chemical injuries of the tissue precede the infection. For example, colonization of exposed subcutaneous tissues with *Staphylococcus aureus* can lead to movement through intact fascial planes with concomitant cellulitis and subsequent bacteremia and systemic infection (Sheagren, 1984), and *Streptococcus pyogenes* can, besides causing pharyngitis, infect soft tissues, lymphatics and the respiratory tract (Bisno, 1991). During systemic infection, several proteins protect *S. pyogenes* against attack by antibodies and the complement system as well as against the uptake and subsequent destruction by professional phagocytes. Expression of protein G (Fahnestock et al., 1986; Filpula et al., 1987; Bjorck and Kronvall, 1984, see 3.3.3), which, similarly to protein A of *S. aureus* (Forsgren and Sjoquist, 1966), binds the Fc portion of antibodies, thus coating the bacteria with antibodies in a way that does not lead to opsonization and recognition by the host immune system. In an other way *S. pyogenes* avoids the host immune response by secreting an enzyme, the streptococcus complement peptidase, which degrades C5a (Scp, Simpson et al., 1990; Chen and Cleary, 1990, see below), a complement factor attracting phagocytes, and by expressing a highly variable surface antigen, protein M (Hollingshead et al., 1987, see 3.3.3., Table 1), which prevents ingestion of *S. pyogenes*. Another highly invasive pathogen, *Yersinia pestis* rapidly disseminates to multiple organ sites (Cornelis, 1992; for a review, see below), as is the case for *Borrelia burgdorferi* and *B. hermsii* causing lyme disease, syphilis and relapsing fever respectively (Coleman et al., 1995).

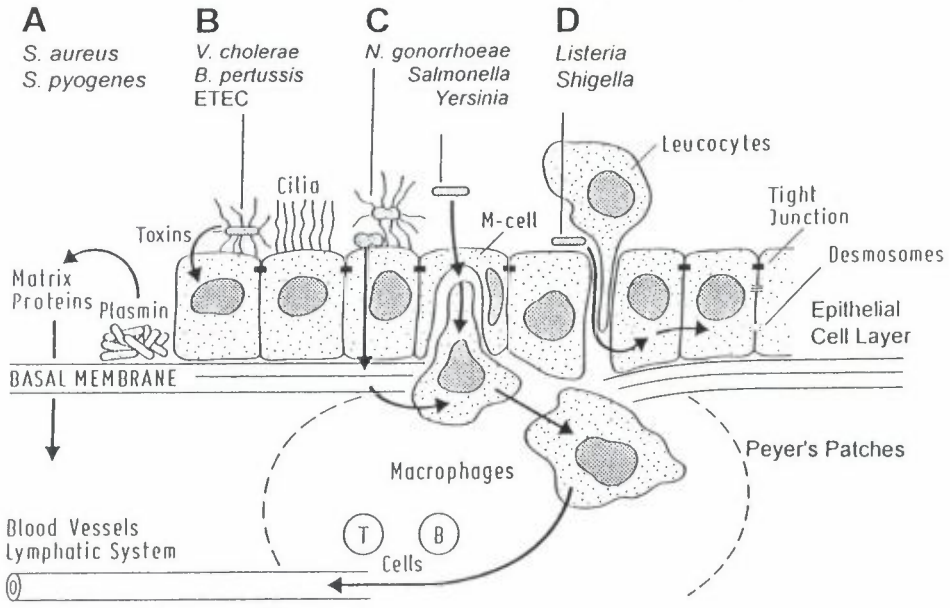


Figure 1. Different groups of pathogenic lifestyles. A: Plasmin-dependent invasion and dissemination; B: Toxin producing pathogens; C: Pathogens which actively cross the epithelial cell layer; D: Infection restricted to the epithelial cell layer. Representatives of each group are indicated.

All of these pathogens and many more like *Neisseria gonorrhoeae, N. meningitidis,* and pathogenic *Escherichia coli* share the ability to infect hosts by penetration of normal tissue barriers (Parkkinen and Korhonen, 1989; Ullberg et al., 1992, Leytus et al., 1989). These consist of cells such as fibroblasts and an organised matrix composed of proteins such as collagen, laminin and fibronectin which restricts the movement of cells between defined compartments (Liotta and Stetler-Stevenson, 1991). Substantial evidence suggests that the bacteria mentioned above can take advantage of the host plasmin system, a main physiological function of which is fibrinolysis of blood clots following successful wound healing (Kwaan, 1992), in order to either escape from a fibrin network deposited by the host to contain a focus of infection or to cross tissue barriers during the course of infection (Lottenberg et al., 1994). Many physiological processes of the host, such as wound healing, tissue remodelling and trophoblast implantation, involve degradation of matrix proteins and the ability of host cells to cross tissue barriers. This is achieved by interaction of these migrating cells with key components of the fibrinolytic system: plasminogen, plasmin,

Table 1. Summary of mechanisms used to generate genetic variation during the infection process of pathogenic bacteria

Mechanism of variation[a]	Organism	Determinant	Element involved	Infection process	Reference
Gen. homologous recombination					
1	*B. hermsii*	*vmp*	Unknown	Immune escape	Plasterk et al., 1985
1B	*B. thuringiensis*	*crylA*	Unknown	Toxin specificity	Caramori et al., 1991
1A	*C. fetus*	SLP	RecA	Immune escape	Dworkin & Blaser, 1996
1	*N. gonorrhoeae*	*pilE*	RecA	Adhesion, immune escape infection stage transition	Haas & Meyer, 1986
1B	*N. gonorrhoeae*	*opa*	RecA	Adhesion, cell tropism immune escape, infection stage transition, invasion	Meyer et al., 1990
1B	*N. meningitidis*	*opa*	RecA	Adhesion, cell tropism immune escape, infection stage transition, invasion	Meyer et al., 1990
Variation via repetitive domains					
2	*A. marginale*	*msp1*	Unknown	Immune escape	Allred et al., 1990
2	*M. hyorhinis*	*vlpA, vlpB, vlpC*	Unknown	Immune escape	Yogev et al., 1991
2	*S. pyogenes*	M protein	Unknown	Phagosytosis resistance	Hollingshead et al., 1987
DNA inversion					
3A	*C. fetus*	*sapA, sapA2*	Unknown	Immune escape	Dworkin & Blaser, 1996
3B	*E. coli*	*e14*	Pin	Unknown	van de Putte et al., 1984
3A	*E. coli*	*fimA*	FimB, FimE, IHF	Adhesion	Abraham et al., 1985
3B	*M. bovis*	*tfp*	Piv	Adhesion, immune escape	Marrs et al., 1988a and b
3B	*M. lacunata*	*tfp*	Piv	Adhesion, immune escape	Rozsa & Marrs, 1991
u	*N. gonorrhoeae*	Unknown	Gcr	Unknown	Rozsa & Fussenegger, subm.

Table 1. Continued

Mechanism of variation[a]	Organism	Determinant	Element involved	Infection process	Reference
u	*S. aureaus*	Unknown	Bin	Antibiotic resistance	Rowland & Dyke, 1988
u	*Shigella* spp.	Unknown	PinB, PinD, PinF	Unknown	Tominaga et al., 1991
3A	*S. typhimurium*	*h2*	Hin, Fis, HU	Motility	Silverman and Simon, 1979
Variation via short repeats					
4B	*B. pertussis*	*bvgS*	poly C	Virulence control	Stibitz et al., 1989
4A	*B. pertussis*	*fim2, fim3, fimX*	poly C	Adhesion, immune escape	Willems et al., 1992
4A	*B. pertussis*	*ptx*	poly C	Adhesion, toxin phase variation	Gross & Rappuoli, 1989
4A	*H. influenzae*	*hifA, hifB*	TA	Adhesion	van Ham et al., 1993
4B	*H. influenzae*	*lex2A*	GCAA	Infection stage transition, serum resistance	Jarosik & Hansen, 1994
4B	*H. influenzae*	*lic1A, lic2A, lic3A*	CAAT	Infection stage transition, serum resistance	Weiser et al., 1989
4B	*H. influenzae*	*tbp*-like	CAAC	Iron acquisition	Hood et al., subm.
4B	*H. influenzae*	*yadA*-like	GCAA	Unknown	Hood et al., subm.
4B	*M. catarrhalis*	*tbp*-like	CAAC	Iron acquisition	Peak et al., 1996
4A	*M. hyorhinis*	*vlpA, vlpB, vlpC*	poly A	Immune escape	Yogev et al., 1991
4B	*N. gonorrhoeae*	*IgtA, IgtB, IgtC*	poly G	Infection stage transition, serum resistance	Gotschlich, 1994
4B	*N. gonorrhoeae*	*opa*	CTCTT	Adhesion, cell tropism, immune escape, invasion	Stern et al., 1986
4B	*N. gonorrhoeae*	*pilC*	poly G	Adhesion, competence. pilus phase variation	Jonsson et al., 1992
4A	*N. gonorrhoeae*	*porA*	poly C	Unknown	Kahrs et al., unpubl.

Table 1. Continued

Mechanism of variation[a]	Organism	Determinant	Element involved	Infection process	Reference
4B	*N. meningitidis*	*icsA*-like	GCAA	Unknown	Jennings et al., 1996
4B	*N. meningitidis*	*lgtA*	poly G	Infection stage transition, serum resistance	Jennings et al., 1995
4B	*N. meningitidis*	*opa*	CTCTT	Adhesion, cell tropism infection stage transition, invasion	Stern & Meyer, 1987
4A	*N. meningitidis*	*opc*	poly C	Adhesion, invasion	Sarkari et a., 1994
4B	*N. meningitidis*	*pilC*	poly G	Adhesion, competence pilus phase variation	Nassif et al., 1994
4A	*N. meningitidis*	*porA*	poly C	Immune escape	van der Ende et al., 1995
4B	*Y. pestis*	*yopA*	poly A	Virulence control	Rosqvist et al., 1988
Variation by DNA modification					
5	*E. coli*	*papA*	Lrp, PapI, PapB Dam, CRP, H-NS	Adhesion, pilus phase variation	van der Woude et al., 1992
Variation by mobile genetic elements					
6	*C. freundii*	Vi antigen	IS1	Immune escape	Ou et al., 1988
6	*N. meningitidis*	*siaA*	IS1301	Infection stage transition phagocytosis resistance serum resistance	Hammerschmidt et al., 1994 and 1996
6	*P. atlantica*[b]	*eps* locus	IS-like	Adhesion	Bartlett et al., 1988

[a]Numbers indicate the type of variation as shown in Fig. 2; u = unknown; [b]non-pathogenic marine bacteria.

plasminogen activators (tissue-type specific plasminogen activator, tPA; urokinase-type plasminogen activator, uPA), plasminogen activator inhibitors (PAI-1, PAI-2) and antiplasmin which directly inhibits plasmin specifically (Liotta and Stetler-Stevenson, 1991; Behrendtsen et al., 1992; Pepper and Montesano, 1990; McNeill and Jensen, 1990). Both, PAIs and antiplasmin belong to a family of serine protease inhibitors called serpins. A plasmin activator which is probably provided by macrophages and monocytes converts plasminogen into its active form, plasmin, by enzymatic cleavage of the zymogen (Vassalli et al., 1992). Besides the degradation of blood clots consisting of fibrin networks and the hydrolysis matrix proteins such as laminin and fibronectin (Salonen et al., 1984; 1985), the serine protease, plasmin, also triggers the activation of enzymes involved in the complement cascade and activates proenzymes such as latent collagenases and other latent metalloproteases (He et al., 1989). Therefore the fibrinolytic cascade is highly regulated by components which inhibit zymogen activation (plasminogen activator inhibitor) or plasmin activity (antiplasmin). Pathogens such as *S. pyogenes*, *S. aureus*, *N. gonorrhoeae*, *N. meningitidis*, *B. burgdorferi*, *B. hermsii* and pathogenic *E. coli* were shown to produce plasmin(ogen) receptors on their surfaces which can bind the zymogen as well as the proenzyme (Kuusela et al., 1992; Kuusela and Saksela, 1990; Ullberg et al., 1992; Coleman et al., 1995; Parkkinen and Korhonen, 1989). Furthermore the plasmin(ogen) binding activity of *E. coli* was found to be dependent on the type of fimbriae expressed (Parkkinen and Korhonen, 1989; Parkkinen et al., 1991). Uropathogenic *E. coli* isolates expressing S or P pili (see 3.1.4, Table 1) show a low affinity for plasmin(ogen), while type 1 fimbriae (see 3.1.2, Table 1) of enterotoxigenic *E. coli* exhibit a high affinity to plasmin(ogen). However, the region of the type 1 fimbriae that interacts with plasmin(ogen) as well as the importance of this interaction in the ability of *E. coli* to adhere to or invade human tissues remains to be elucidated. It seems to be a common feature of all surface bound plasmin(ogen) that it is protected from physiological inhibition by the host serpins. However, most of these bacteria are dependent on host plasmin activators, tPA and/or uPA for the generation of active plasmin, except *S. pyogenes* (streptokinase, Castellino, 1979), *S. aureus* (staphylokinase, Matsuo et al., 1990), *E. coli* (Leytus et al., 1981) and *Y. pestis* (Pla, Sodeinde et al., 1992), which produce and secrete their own plasminogen activators. These plasminogen activators usually form a 1:1 stoichiometric complex with plasmin(ogen) which then converts plasminogen to plasmin (Castellino, 1979). These active complexes are either regulated (staphylokinase) or not (streptokinase) by host serpins (Lijnen et al., 1991).

In the case of *Y. pestis*, Pla not only functions as plasmin activator but represents a central virulence factor since it also degrades C3b and C5a of the complement system, thereby alleviating phagocytosis by macrophages and

neutrophiles as well as the assembly of the membrane attack complex of the complement system (MAC) (Sodeinde et al., 1992). Additionally, it modulates the Yop concentration by degradation and may also play a role in insect-mediated transmission of the bacteria (Sodeinde and Goguen, 1989, see C2.).

It is an interesting parallel to these bacterial systems that metastatic spread of transformed eukaryotic cells has also been associated with the ability of invasive tumor cells to produce plasminogen activators (uPA) as well as surface receptors for plasmin(ogen) (Ossowski, 1992). Given such parallels between the interaction of prokaryotic and eukaryotic cells with the plasminogen system it may form a basis for a conserved mechanism used by cells to cross tissue barriers a prerequisite for bacterial pathogens to establish systemic infections. However, there exist other mechanisms to desintegrate tissue barriers such as the breakdown of collagen by collagenases (*Clostridia*, Bond and van Wart, 1984) or the breakdown of hyaluronic acid, a polysaccharide that functions in the body as a tissue cement, by hyaluronidases produced by streptococci, staphylococci, pneumococci and certain *Clostridia* spp. (Hynes and Ferretti, 1994).

B. Toxin producing pathogens (Fig. 1B)

Exotoxins are toxic proteins produced by some Gram-negative and Gram-positive bacteria (see Sears and Kaper, 1996 for a review). Many of these toxins are excreted into the surrounding, but some are released only when the bacteria lyse. There are three general types of exotoxins. One type consists of membrane-disrupting toxins which disrupt host cell membranes either by forming channels, i.e. listeriolysin (LLO, *Listeria monocytogenes*, Bielecki et al., 1990), pneumolysin (PLO, *Streptococcus pneumoniae*, Rubins et al., 1992), and presumably IpaB (*Shigella flexneri*, High et al., 1992, see chapter D), by lysing red blood cells (hemolysins; *E. coli* HlyA, Konig et al., 1994; *Serratia marcescens* ShlA, Schonherr et al., 1994; *Clostridia* perfringolysin O, Awad et al., 1995; *Staphylococci*, Freer et al., 1968; *Streptococci* streptolysin O, SLO, Bhakdi et al., 1985; *Bordetella pertussis*, CyaA, Glaser et al., 1988) or by hydrolysing phospholipids in the membrane i.e phospholipases of *Clostridium perfringens* (Awad et al., 1995; Sears and Kaper, 1996). Another type is superantigens which bind to the major histocompatibility complex (MHC) Class II on antigen presenting cells (APC) and to T cell receptors to force an unnatural association of APCs and T cells. This results in the release of high levels of interleukin 2 and ultimately causes other cytokines to be released finally leading to a septic shock (Hewitt et al., 1992; Schlievert, 1993 for a review). The best characterized superantigens are the toxic shock syndrome toxin (TSST-1, Schlievert, 1983) of *S. aureus* and Spe, an endotoxin of *S. pyogenes* (Lee and Schlievert, 1991). The toxic shock syndrome TSS caused by *S. aureus* first appeared in the late 1970s and was found to be associated with super-

absorbent tampons (Schlievert, 1983). In the 1980s a high-mortality TSS-like disease (toxic shock-like syndrome, TSLS) appeared, which was caused by the same toxin associated earlier with scarlet fever: Spe (Lee and Schlievert, 1991). The third type is A-B exotoxins which contain one portion (B, one or five subunits) that binds to the host cells and a second portion (A, one subunit) that is enzymatically active (Silverstein and Steinberg, 1990). After the binding of the B subunit(s) to a host cell receptor, the A subunit is translocated through the host cell membrane into the cytoplasm where it exerts its toxic effect. Diphtheria toxin (*Corynebacterium diphtheriae*) ADP-ribosylates a host elongation factor which causes the arrest of host protein synthesis (Honjo et al., 1968). The shiga toxin (*Shigella disenteriae*) and probably also the shiga-like toxin of enterohemorrhagic *E. coli* (EHEC) achieve the same result by cleaving the host cell's rRNA (Endo et al. 1988). Furthermore, tetanus toxin (*Clostridium tetani*) blocks the activity of a nerve factor that allows the relaxation of a muscle, and the botulin toxin (*Clostridium botulinum*) prevents the release of acetylcholine (Schiavo et al. 1992). Toxins of *Vibrio cholerae* (cholera toxin, Mekalanos et al., 1979; Kaper et al., 1995), enterotoxigenic *E. coli* (ETEC, heat-stable and heat-labile (ST and LT) enterotoxins, Carpick and Gariepy, 1993; Spangler, 1992) and *B. pertussis* (pertussis toxin, Ptx, the etiological agent of whooping cough, Weiss and Hewlett, 1986), either activate guanylate cyclase (ST, Carpick and Gariepy, 1993) or ADP ribosylate G proteins (all others) causing a disruption of the control of cyclic AMP and resulting in the cases of *V. cholerae* and ETEC, in the secretion of fluid and electrolytes from the intestinal epithelia (Spangler, 1992; Kaper et al., 1995). Yet another mechanism to increase the intracellular cyclic AMP concentration of host cells has been shown for the *B. pertussis* invasive adenylate cyclase (CyaA), an A-B toxin which overrides the corresponding host components regulating cyclic AMP levels (Glaser et al., 1988; Hewlett et al., 1989). Besides its toxic effect, Ptx is also a main adhesin of *B. pertussis* (Saukkonen et al., 1992), and like may virulence factors of this pathogen, is subject to regulation by the phase-variable BvgS (Stibitz et al., 1988, see 3.1.3.). *B. pertussis* preferentially binds to cilial cells and kills them by the help of the tracheal cytotoxin. Thereby *B. pertussis* prevents being swept out of the airway. The impairment of cilal cells seems to be a common theme of tracheal pathogens (Kaslow and Burns, 1992; Johnson and Inzana, 1986; see chapter C2.).

Membrane disrupting toxins can be of selectional advantage to the pathogen since they could allow the sequestering of iron (hemolysins), the escape of phagocytic vesicles (listeriolysin, IpaB), the acquisition of nutrients by disintegrating host cells (phospholipases) or disrupting the epithelial cell layer in order to reach deeper tissues (pneumolysin). For example, *S. pneumoniae* colonizes the nasopharynx and, after having bypassed the clearance mechanism of the upper airway, it reaches the lung where pneumolysin, which shares amino

acid homology with listeriolysin (*L. monocytogenes*) and streptolysin (*S. pyogenes*), binds to the cholesterol of epithelial cells and disrupts the membrane (Rubins et al., 1992). Thereby *S. pneumoniae* gains access to the bloodstream and can, since its polysaccharide capsule protects it from serum components, reach the brain where it subsequently causes meningitis (DeVelasco et al., 1995, for a review). Besides the antiphagocytic effect of the pneumococcal capsule, pneumolysin also inhibits the respiratory burst of macrophages (Nandoskar et al., 1986).

Contrary to membrane-disrupting toxins, it is still not clear what benefits the bacteria derive from adherence to mucosal surfaces and the subsequent production and secretion of A-B toxins (Fig. 1), which belong to the most lethal molecules known (1 mg of botulin toxin is sufficient to kill 10^6 rabbits). Given the fact that, unlike the cholera toxin, many genes encoding these toxins are located on temperate bacteriophages (diphtheria toxin, botulin toxin and Spe of *S. pyogenes*) or on plasmids (tetanus toxin, *E. coli* and staphylococcal enterotoxins) rather than on the bacterial chromosome may indicate a selectional force maintaining such phages and plasmids (Silverstein and Steinberg, 1990). The question of the benefit of A-B toxins for the bacteria is especially perplexing in the case of cholera toxin, because the effect of diarrhoea produced by the toxin is to wash the bacteria out of the intestine. It is now gaining increasing acceptance that what strikes the humans as the most important aspect of the toxin action may not be the effects "sought" by the bacteria. It is postulated that these bacteria are aiming for a long-term colonization of the intestine and that the narrow evolutionary window of this host-pathogen interaction we describe today as cholera, is rather reflecting an unsuccessful colonization attempt of bacteria which are not yet optimally adapted to the intestinal microenvironment. This is supported by the fact that only minor fractions of human populations which have long been in contact with *V. cholerae* develop full-blown cholera during an epidemic. The production of toxins may then be useful for colonization by mediating adherence to mucosal surfaces. In fact, the pertussis toxin serves such a dual function being both a toxin as well as an adhesin (Saukkonen, et al., 1992; Weiss and Hewlett, 1986) and there is considerable amino acid homology found between enterotoxigenic *E. coli* (ETEC) K99 pilin, the CFAI (colonization factor antigen) fimbrial subunits of ETEC, which both mediate adhesion, the B subunits of cholera toxin and the heat-labile (LT) toxin of enterotoxigenic *E. coli* (Jacobs et al., 1986). Another possibility is that toxins may be maintained by coincidental selection: they presumably exist because they have neutral or fitness-enhancing effects in situations where they do not cause disease but rather unexpectedly become virulence factors upon accidental contact with the host (Read, 1994). However, it seems to be a generally accepted concept that successful pathogens such as those described in the following chapter have evolved ways to limit their

virulence, so that they remain virulent enough to bypass host defences but not virulent enough to kill most hosts.

A novel type of antigenic bacterial toxin which does not fit in the groups mentioned above is the vacuolating enzyme, VacA, of *Helicobacter pylori*, which induces vacuoles in gastric epithelial cells, subsequently leading to cell death (Cover et al., 1991; Schmitt and Haas, 1994). In conjunction with other virulence factors such as the lipopolysaccharide (LPS), a heat-shock protein (hsp) which can elicit T cells and antibodies that crossreact with human hsp60, and the urease which produces a rather neutral pH in the immediate bacterial surroundings by converting urea to ammonia and carbon dioxide, the vacuolating cytotoxin is the main component in the destruction of the gastric mucosa and ulcer formation in humans (Lee et al., 1993; Blaser, 1992).

C. Pathogens which actively cross the epithelial cell layer (Fig. 1C)

The bacterial pathogens of this group are able to actively cross the host epithelial cell barriers, subsequently infect subepithelial tissue, and reach the blood or the lymphatic vessels from where they disseminate in the whole body (*N. gonorrhoeae*) to finally grow in the spleen and liver (*Salmonella* spp.), the lymphnodes and the lung (*Yersinia* spp.) or they cross the blood-brain barrier and colonize the brain and the spinal fluid (*N. meningitidis, Haemophilus influenzae, S. pneumoniae*). Whereas many pathogens can gain access to deeper tissues and even the bloodstream via lesions in the epithelial cell layer, the members of this group such as *Yersinia* spp., *Salmonella* spp. and *Neisseria* spp., which are among the best studied pathogens, harbour a genetic infection program which enables these bacteria not only to proceed stage by stage with the infection but also to withstand the various host defence systems and recurrent changes in the different microenvironments which are subsequently encountered during the course of infection.

C1. *Neisseria gonorrhoeae*

N. gonorrhoeae starts its infection process by loosely attaching to the epithelial cell layer. This process is mediated by type 4 pili, thin filamentous appendages protruding from the bacterial cell surface, which are produced by an increasing number of bacterial pathogens (for an overview see Hobbs and Mattick, 1993; Strom and Lory, 1993; Iredell and Manning, 1994) such as *V. cholerae, Pseudomonas aeruginosa* (see chapter 2) and *Moraxella lacunata* and *M. bovis* (see 3.1.2.). The latter two are closely related to *N. gonorrhoeae* and cause infectious human or cattle keratoconjunctivits, a highly contagious eye disease (Marrs et al., 1988a and b; Rozsa and Marrs, 1991). Although the gonococcal pilus subunit, PilE, exhibits some adhesive properties by itself (Virji and Heckels, 1984; Rudel

et al., 1992), the minor pilus protein, PilC – which is also involved in pilus assembly and DNA transformation (Jonsson et al., 1992; Rudel et al., 1995a; Fussenegger et al., submitted, see 3.1.3.) – was recently identified as adhesin associated with the tip of the pilus (Rudel et al., 1995b), similar to PapG, SfaS and FimH of the P and the S pilus and type I fimbriae of uropathogenic *E. coli* respectively (Lindberg et al., 1987; Moch et al., 1987; Abraham et al., 1988). As a common theme in bacterial adhesion the loose pilus-mediated attachment is further strengthened and becomes more "intimate" by the binding of bacterial surface proteins to carbohydrated moieties displayed on the host cell surface. In *N. gonorrhoeae* and *N. meningitidis* such an intimate adhesion is mediated by one of several phase-variable outer membrane proteins (Opa) on the bacterial side (Kupsch et al., 1993) and the heparan sulfate side chains of a surface proteoglycan on the target cell side (van Putten and Paul, 1995). One Opa (Opa_{30}) was also shown to mediate invasion of epithelial cells (Makino et al., 1991), but additional invasion determinants participate in this process (Kahrs et al., 1994; Fussenegger et al., 1996b). The mutants of these determinants remain to be characterized (Kahrs et al., 1994). The invasive behaviour of *N. meningitidis* seems to be similar to *N. gonorrhoeae*, although this pathogen starts its infection in the nasopharynx by pilus- and PilC-mediated, loose contact, followed by adherence and invasion of the epithelial cells mediated by Opa and Opc (Virji et al., 1993a; Sarkari et al., 1994). Little is known about the sequential steps following invasion of epithelial cells, but *N. gonorrhoeae* seems to traverse epithelial cells and comes into contact with professional phagocytes such as macrophages and polymorphonuclear neutrophils (PMN) in the subepithelial tissue. Whether *N. gonorrhoeae* survives or is killed by phagocytic cells is still under debate. However, experiments showing that the neisserial PI, a pore in the outer bacterial membrane which allows passive diffusion of low-molecular-weight nutrients, has an additional function inside phagocytic cells favour the idea of gonococcal survival inside these cells. PI can form pores in eukaryotic cells when purified PI is exogenously added to cultured epithelial cells *in vitro*, and *in vivo* transfer of PI into eukaryotic membranes via bacterial membrane vesicles was also reported. Such insertions could prepare the bacterial uptake mechanism, probably either by alleviating induction of the phagocytic defence mechanisms (i.e. oxidative burst) or by preventing the phagosome-lysosome fusion by collapsing the membrane potential (Weel et al., 1991; Weel and van Putten, 1991). Furthermore, PI also binds the eukaryotic calcium-binding protein, calmodulin, and interferes in this way with host-cell signalling. Such interference was also shown to inhibit either the phagosome-lysosome fusion or the oxidative burst after such a fusion. *N. gonorrhoeae* which escape from phagocytic cells upon their lysis then enter the bloodstream where serum resistance, the resistance to the killing by the MAC, is a prerequisite for strains that cause systemic infection.

The carbohydrated portion of the LPS is involved in activation of the alternative complement pathway by both binding factor C3b and ingestion by phagocytes via their C3b receptors, and it serves as a nucleation site for formation of the MAC by binding complement factor C5b. It has long been demonstrated that gonococcal and meningococcal populations display structural heterogeneity of their LPS (Schneider et al., 1988), with long LPS species predominantly isolated from the blood (Jones et al., 1992) and short LPS preferentially expressed by nasopharyngal isolates (Broome, 1986). The major difference between the variant LPS molecules is the presence of additional carbohydrate residues in the longer forms. These can be externally modified by a membrane-associated, bacterial sialyltransferase using host-derived or endogenous cytidine 5'-monophospho-*N*-acetyl neuramic acid (CMP-NANA) as the sialyl group donor (van Putten, 1993). The relevance of the LPS phase transitions has recently been elucidated (van Putten, 1993) and appears to lie in the variable quantities of sialic acid incorporated in the different forms of LPS. A low sialylation phenotype expressing short LPS, such as that typically found early in the infection (Schneider et al., 1991), enables entry of the bacteria into mucosal cells but makes them susceptible to bactericidal activity. In contrast, highly sialylated bacteria expressing long LPS are incapable of entering epithelial cells but are resistant to phagocytosis and killing by antibodies and complement since they mimic host cells which contain sialic acid residues as an ubiquitous surface component, thus allowing persistence of infection. The attachment of sialic acid to the LPS prevents formation of the host C3 convertase, similar to sialic acid containing capsules (see below and 3.3.4.), and the length of the LPS chains prevent effective MAC formation, probably because it is too far from the bacterial outer membrane to exert a bactericidal effect. A similar effect has been shown for LPS O-antigen protection of *Salmonella* (Jimenez-Lucho et al., 1990; see chapter C3). Thus, depending on the degree of sialylation the bacteria are either adapted to the extracellular environment and are capable of resisting humoral immune mechanisms, or they become sensitive to bactericidal activities but can readily invade epithelial cells via Opa-mediated cellular interactions.

Interestingly, sialic acid is also found as a modification of the meningococcal LPS. Substitution of the terminal galactose residue of the LPS with sialic acid depends on the availability of endogenously synthesized or exogenous CMP-NANA. The mechanisms resulting in LPS length polymorphisms were found to be nearly identical in *N. gonorrhoeae* and *N. meningitidis* (Gotschlich, 1994; Jennings, 1995; see 3.3.2.; Table 1). In contrast to *N. gonorrhoeae*, *N. meningitidis* is able to express a capsule consisting of homopolymers of sialic acids (Frosch et al., 1989). This high amount of sialic acid expressed on the surface of these bacteria is associated with their virulence as it mediates resistance to both phagocytosis and to complement-mediated killing via alternative pathway activation. Similar to

length variation of gonococcal LPS, the meningococcal capsule can be switched off, thus enabling Opa- and Opc-mediated invasion and passage through the mucosal epithelium. This is followed by re-expression of the cell-surface sialic acids to resist the host's immune defence which is a prerequisite for persistence in the bloodstream and crossing the blood-brain barrier. Such a change in capsule expression is due to a novel mechanism (see 3.3.4.) involving *siaA*, the essential sialic acid biosynthesis gene which, besides capsule expression, influences also endogenous LPS sialylation (Hammerschmidt et al., 1996; Table 1).

LPS is also an important virulence factor in two other pathogenic species both of which will be described later for their capability for phase and antigenic variation: *P. aeruginosa* (see chapter 2) and *H. influenzae* (see 3.3.2.).

Similar to *N. meningititidis*, an interesting correlation has been observed in *P. aeruginosa* between LPS transition from a long and negatively charged O antigen (B form) to a shorter rather neutral form (A form) and transitions from a noncapsulated (nonmucoid) to a capsulated (mucoid) form. *P. aerugniosa* grows – often with a fatal outcome – in the lung of cystic fibrosis patients, where it switches from the nonmucoid (B form of LPS) to the encapsulated (A form of LPS). The mechanism of these coupled transitions is postulated to be due to reversible expression of a sigma factor (Govan and Harris, 1986; Hancock et al., 1983; DeVries and Ohman, 1994, see chapter 2). Whereas the mucoid phenotype, which is the result of overproduction of the exopolysaccharide, alginate, appears to confer both increased resistance to phagocytosis (Baltimore and Mitchell, 1982) and adherence that permits the formation of microcolonies in the lung (Lam et al., 1980), the neutral type A LPS renders *P. aerugniosa* essentially resistant to most antibiotics (Kadurugamuwa et al., 1993). LPS is also important for *H. influenzae*, a common commensal of the human upper respiratory tract to which it is highly adapted. However, it can occasionally become a highly virulent, invasive strain causing among other things meningitis and pneumonia. It was shown that *H. influenzae* LPS enhances bacterial survival in the nasopharynx and facilitates the invasion of this organism across cellular barriers by LPS-caused loss of ciliary activity and disruption of ciliated epithelial cells (Johnson and Inzana, 1986). The observed variability of the sugar composition of *H. influenzae* LPS as manifested in different serotypes was shown to be influenced by the *lic* loci that are required for the expression of phase-variable oligosaccharide epitopes (Weiser et al., 1990). Proteins encoded by these *lic* loci were not only shown to be responsible for the antigenic variation of the LPS, but they also alter the level of expression (Weiser et al., 1989). Interestingly, a higher level of LPS expression correlates with greater resistance to serum-factor induced bacteriolysis (Kuratana et al., 1990, see 3.3.2.).

C2. Yersinia species

Three *Yersina* spp. cause disease in humans: *Yersinia enterocolitica, Y. pseudotuberculosis* and *Y. pestis* (Cornelis, 1992). *Y. enterocolitica* and *Y. pseudotuberculosis* have – after ingestion of contaminated food – to cross the gastrointestinal mucosa in order to infect the underlying tissue where these infections usually remain localised. *Y. pestis* does not have to penetrate a body surface on its own since it is injected by an insect bite. Once inside the body, *Y. pestis* rapidly spreads to the nearest lymph nodes where it is ingested by fixed macrophages inside which it can survive and proliferate until the macrophage eventually reaches the lung and releases the bacteria that parasitize the lung macrophages and spread to other hosts via aerosols.

Y. enterocolitica and *Y. pseudotuberculosis* both have an affinity for Peyer's Patches and probably transit the mucosa through M cells (Fig. 1), which are naturally phagocytic cells that would ordinarily engulf the adherent bacteria and pass them to the underlying lymphoid cells of Peyer's Patches for destruction (Grützkau, et al., 1990). Three proteins are involved in *Yersinia* adherence to and invasion of epithelial cells: Ail, (attachment-invasion locus, Miller et al., 1989), YadA (*Yersinia* adherence, Heesemann and Grüter, 1987; Bliska et al, 1993) and the invasin (Inv, Pierson and Falkow, 1990). The two latter were found to bind to integrins, as seems to be the case for *S. flexneri* (Ipa proteins, see chapter D) and other invasive intestinal pathogens (i.e. pathogenic *E. coli*) (Isberg and Leong, 1990). The observation that integrins are located on the basolateral surface of mucosal cells raises the question of whether invasins are involved in the initial invasion of the mucosal surface or in some subsequent step such as infection of the mucosal layer from below, which may contribute to the carrier state (Tran Van Nhieu and Isberg, 1993). Furthermore, many cells of the immune system, including phagocytes express integrins on their surfaces and invasins may allow the bacteria to enter phagocytes in a way that either bypasses normal phagosome formation or gives the bacteria time to escape from the vesicle in which they are ingested before fusion with the lysosome occurs (see chapter D). Many bacteria that produce integrin-binding invasins appear to use M cells for invasion despite the fact that the natural role of M cells is to sample antigens and not to serve as a "highway" for invading bacteria.

Both *Y. enterocolitica* and *Y. pseudotuberculosis* are killed by PMNs. Thus, both bacteria have developed an antiphagocytic strategy based on the plasmid encoded proteins called *Yersinia* outer membrane proteins (Yops) (Strahley et al., 1993). The excretion of Yops involves a number of proteins among them the Ysc (*Yersinia* secretion) which probably process and excrete Yops in a manner similar to processing and excretion of pilin subunits and toxins (Michiels et al., 1990; Fussenegger et al., submitted; Hobbs and Mattick, 1993; see chapter D). There are two functional classes of Yops: those that interfere with signal transduction in

host cells (i.e. the ability of phagocytes to respond to environmental signals) and those affecting the host cell cytoskeleton. Although most Yops are secreted into the medium some, such as Yop E and Yop H, seem to be directly injected into the host cell (Rosqvist et al., 1994). YpkA (*Yersinia* protein kinase) and YopH (a phosphatase) "reprogram" host cell signalling and may directly prevent phagocytes from migrating towards and phagocytosing bacteria (Galyov et al., 1993; Guan and Dixon, 1991). YopM prevents cell signalling in general and thereby prevents the release of inflammatory mediators which would stimulate phagocytes and other cells of the immune system (Leung and Straley, 1989; Reisner and Straley, 1992). On the contrary, YopE destroys the cell cytoskeleton. This mechanism may function to kill phagocytes as well as other eukaryotic cells encountered during infection (Rosqvist et al., 1994).

All three *Yersinia* spp. are serum resistant. While serum resistance in *Y. enterocolitica* and *Y. pseudotuberculosis* is mediated by YadA and Ail (Balligand et al., 1985; Martinez, 1989), *Y. pestis* takes advantage of its plasminogen activator PlaI (see chapter A) which degrades C3b and C5a and prevents formation of the MAC and chemoattraction of phagocytes (C5a, see *S. pyogenes*, above) (Sodeinde et al., 1992). Furthermore, PlaI degrades Yops and can thereby regulate the Yop concentration so that it is high enough to protect from PMNs and low enough to allow uptake by the circulating monocytes or macrophages which *Y. pestis* parasitizes (Sodeinde and Goguen, 1989).

C3. Salmonella species

Another food borne disease of group C pathogens is caused by *Salmonella* spp., mainly *Salmonella typhimurium* and *S. typhi* the causative agent of typhoid fever. As for the *Yersinia* spp., *Salmonella* spp. seem to enter the mucosal cells via M-cells of the Peyer's patches. Upon contact with bacteria, human epithelial cells show rearrangement of actin and a splash-like deformation (ruffling) of their membrane at the contact side mediated by the invasion gene products InvA-H (Galan and Curtiss III, 1989; Galan et al., 1992; Kaniga et al., 1994; Eichelberg et al., 1994). It could be shown that the binding of *Salmonella* to the host stimulates the epidermal growth receptor and triggers a cascade of phosphorylating and dephosphorylating reactions which eventually lead to actin rearrangements and the uptake of the bacteria (Galan et al., 1992; Pace et al., 1993). Although there are significant differences between the ways in which *Salmonella* spp., *Yersinia* spp., and *Shigella* spp. interact with their hosts, it becomes more and more evident that all of these pathogens share a dedicated protein secretion system required to present or deliver determinants that are essential for triggering bacterial uptake (Maurelli, 1994; Hobbs and Mattick, 1993). These pathogens not only share homologous determinants of their secretion machineries such as Mxi (membrane expression of invasion) of *Shigella* spp. (Sansonetti, 1992), Ysc (*Yersinia* secretion)

of *Yersinia* spp. (Michiels et al., 1990), and Inv of *Salmonella* spp. (Eichelberg et al., 1994) but the targets of this type of secretion system are also highly homologous. These include the invasion plasmid antigens of *Shigella* spp. (Ipa proteins, Sansonetti, 1992; see chapter D), the *Yersinia* outer membrane proteins (Yops, Strahley et al., 1993) and the recently reported *Salmonella* invasion proteins, SipB and SipC which show high homology to the corresponding invasins IpaB and IpaC required by *Shigella* spp. for entry into epithelial cells (Kaniga et al., 1995; see chapter D). This protein secretion system is also present in other bacterial pathogens, including *P. aeruginosa* as well the plant pathogens of the *Aeromonas* spp., and *Xanthomonas* spp., and is also a part of the type 4 pilin assembly apparatus (Fussenegger et al., submitted, Hobbs and Mattick, 1993; Van Gijsegem et al., 1993).

After invasion, *Salmonella* remains in the phagosome and multiplies there before a release of the bacteria occurs after lysis of the host cell. *Salmonella* then disseminates in the bloodstream and multiplies as facultatively intracellular pathogen mainly in macrophages of the spleen and the liver from where it is released into the bloodstream in large numbers. Eventually, these bacteria move from the liver to the gall bladder and reach the intestine again for a further round of infection. In order to survive inside macrophages, *Salmonella* have developed a set of at least 40 proteins which contribute to the survival in phagocytes by conferring resistance to reactive forms of oxygen and defensins (toxic host peptides that kill bacteria) (Kaufmann and Flesch, 1992). As with *Neisseria* spp. and *H. influenzae*, LPS is involved in serum resistance: longer O side chains of the *Salmonella* LPS cause the MAC to form far enough to prevent it from interacting productively with the bacterial outer membrane and provided that this long LPS contains sialic acid prevents the formation of C3 convertase (see above; Jimenez-Lucho et al., 1990). Furthermore, the outer membrane protein Rck (resistance to complement killing) prevents the formation and insertion of fully polymerised, tubular C9 complex (the last step in MAC formation) into the bacterial outer membrane. Another surface structure Vi antigen which is expressed by the most virulent *S. typhi* strains was suggested to be an important virulence factor and explaining to some extent why *S. thyphi* is much more virulent than other *Salmonella* spp. although this is still somewhat controversial (Johnson and Baron, 1969). Vi antigen is a capsular polysaccharide composed of N-acetylglucosamine uronic acid. Capsular polysaccharides are usually produced by bacteria to prevent phagocytosis, however, *S. typhi* appears to survive within phagocytes (Cross, 1990). Nevertheless, capsule proteins like Vi antigen could also function as scavengers of reactive forms of oxygen, one killing mechanism of phagocytes (Chan et al., 1989). Whereas the expression of *Salmonella* Vi antigen is relatively stable, the one expressed by *Citrobacter freundii*,

an opportunistic human pathogen is subject to phase variation (Ou et al., 1988; see 3.3.4., Table 1)

It is interesting that many other pathogens besides *Salmonella* spp. such as *Mycobacterium tuberculosis, M. leprae, L. monocytogenes* (see chapter D), *Brucella abortus, Legionella pneumophila* and the protozoan parasite *Leishmania donovani* use professional phagocytes, macrophages, as their preferred habitat, cells which are normally responsible for clearing an invading pathogen (Hahn and Kaufmann, 1981; Kaufmann and Flesch, 1992 for a review). These pathogens developed strategies to withstand the killing potential of macrophages which includes, oxidative burst (Babior, 1984), production of nitrogen intermediates (Liew and Cox, 1990), acidification of the phagosome-lysosome fusion (Horwitz, 1988), the release of defensins and the limitation of iron by down regulation of the transferrin receptors (Ganz et al., 1988; Byrd and Horwitz, 1991). Iron limitation is a general problem for pathogens which is alleviated by either secreting iron chelating proteins (siderophores) or by binding human iron binding proteins such as transferrin and lactoferrin. The transferring binding protein Tbp, i.e., is an important virulence factor, which competes with the host cell iron sequestering machinery (Meyer, et al., 1994; Payne and Lawlor, 1990, for a review; see 3.3.2.).

M. tuberculosis and *M. leprae* enter macrophages via C3b receptors without inducing an oxidative burst (Horwitz, 1988; Clemens, 1996) and *M. leprae* as well as *Leishmania* additionally express a surface protein which scavenges oxygen radicals (Chan et al., 1989). *S. typhimurium* and *M. tuberculosis* inhibit phagosome acidification and phagosome-lysosome fusion by secretion of ammonium chloride (Hart et al., 1991), *Leishmania* and some *salmonellae* are resistant to lysosomal enzymes (Kaufmann and Reddehase, 1989) and *Listeria* spp. and *Shigella* spp. simply escape from the phagosome-lysosome fusion by evasion into the cytoplasm (Bielecki et al., 1990; Kaufmann and Flesch, 1992; High et al., 1992; see chapter D). Another pathogen *L. pneumophila* seems to find within macrophages a similar environment as inside its normal host, free-living amoebae. *L. pneumophila* which induces uptake into macrophages by the Mip protein (Hacker and Fischer, 1993) also prevents phagosome-lysosome fusion and produces enzymes such as acid phosphatases, phospholipase C, protein kinases and superoxiddismutase all of which can enhance the survival within phagocytes.

The survival within phagocytes is important for the dissemination of the pathogen via the bloodstream to any site of the body abusing these blood cells as carriers. Even bacteria such as *N. gonorrhoeae* and *N. meningitidis* which seem not to parasitize professional phagocytes evolved a mechanism which allows, by the help of the pore protein PI, entry into PMNs or macrophages without triggering the oxidative burst (Haines et al., 1991; Lorenzen et al, unpublished). *N.*

meningitidis is thought to be carried by phagocytes to its final destination: the blood-brain barrier.

D. Infection restricted to the epithelial cell layer (Fig. 1D)

In contrast to pathogens of group C for which the epithelial cell layer represents only a stage they cross in order to reach deeper tissues and/or to cause systemic infection by spreading via the blood and lymphatic system, pathogens such as *L. monocytogenes, Shigella* spp., enteroinvasive *E. coli* (EIEC) and enteropathogenic *E. coli* (EPEC) only invade the mucosal cell layer and the immediate underlying tissue thereby killing the invaded cells. EPEC strains adhere to the apical surface of mucosal cells via a bundle-forming pilus (Giron et al., 1991) which is similar both in structure and amino acid sequence to the type 4, toxin co-regulated pilus (Tcp) of *V. cholerae* (Iredell and Manning, 1994), but the EPEC pilus is not identical to the ETEC bundle forming pilus called longus (Giron et al., 1994). Following pilus-mediated attachment, closer intimin-mediated contact of EPEC with epithelial cells (Jerse et al., 1990) triggers a signal transduction event resulting in a higher intracellular Ca^{2+} concentration (Foubister et al., 1994) and consequently the phosphorylation of host proteins (Rosenshine et al., 1992) which could eventually lead to the deformation and destruction of microvili. Another consequence of signal transduction is the formation of a pedestal-like structure at the contact point in the host cell cytoplasm composed of a dense mat of actin fibers (Knutton et al., 1989). However, it remains unclear whether invasion of the mucosa by EPEC strains actually occurs in human disease. Certainly, EPEC strains are not as invasive as *Shigella* spp. Recently, it has been shown that the induction of intracellular Ca^{2+} concentration by adherent EPEC leads to the disruption of the tight junctions of epithelial cell monolayer (Spitz et al., 1995). This could dissolve the epithelial cell layer and could account, together with the overall subversion of cellular signal transduction systems, for the diarrhea and other symptoms of EPEC infections without the need for actual invasion. In contrast to EPEC strains, *L. monocytogenes, Shigella* spp. and EIEC are not able to invade epithelial cells from the apical side. Although very little is known on the molecular basis of virulence of EIEC strains, the existing data as well as the overall symptomatic similarity to shigellosis suggests that the characteristics of EIEC virulence is virtually identical to those of *Shigella* spp. (Hsia et al., 1993). As for *Yersinia* spp. and *Salmonella* spp., *Shigella* spp. are unable to invade the apical pole of colonic cells since the receptor of the Ipa proteins (IpaB-D) are likely to be integrins which are only found on the basal side of epithelial cells (see above). Therefore, the very first invasion appears to occur via M cells of the Payer's patches. The subsequent ability of *Shigella* spp. to burrow between and beneath the epithelial cell layer

and the interjunctional invasion and destruction of the epithelium is primarily due to the immigration of leukocytes, particularly PMNs that destroy cohesion of the epithelial layer (Perdomo et al., 1994). Similarly, *L. monocytogenes* is thought to infect the epithelial cell layer or it can enter intestinal crypt cells, which are the only undifferentiated mucosal cells. However, in contrast to shigellae, *L. monocytogenes* only expresses a single invasin called Internalin (InlA, Dramsi et al., 1993) which is structurally analogous to the M protein of *S. pyogenes* (Fischetti et al., 1990; see 3.3.3.). Since InlA also appears to bind to a complement receptor on macrophages, *L monocytogenes* can also invade and parasitize these professional phagocytes.

After the uptake of *Shigella* spp., EIEC or *L. monocytogenes*, extensive reorganization of host actin filaments, similar to group C pathogens, continue to occur around the phagocytic vesicle that contains the bacteria. However, *L. monocytogenes, Shigella* spp. and EIEC produce extracellular proteins, i.e. listeriolysin (LLO, *L. monocytogenes,* Bielecki et al., 1990) and IpaB (*S. flexneri,* High et al., 1992), that cause the rupture of the vesicle (see chapter B). Consequently, the bacteria escape into the host cell cytoplasm where they multiply rapidly and exhibit a type of movement which consists of the polymerization of actin filaments at one end of the bacteria, creating "comet-like tails" and propelling the bacteria through the host cell cytoplasm (intracellular spread, IcsA of *Shigella* spp., which shows a gonococcal IgA protease-like secretion mechanism, Lett et al., 1989; Goldberg and Theriot, 1995; ActA of *L. monocytogenes,* Kocks et al., 1992). They eventually come in contact with the adjacent cell membrane and push into the adjacent host cells, forming protrusions from which they eventually escape (IcsB of *Shigella* spp., Allaoui et al., 1992; and PlcB a phospholipase of *L. monocytogenes*, Vazquez-Boland et al., 1992). For *Shigella* spp. an organelle-like movement (Olm) along actin filaments was described in addition to the IcsA-mediated spread (Vasselon et al., 1991).

II. Phase and Antigenic Variation

1. The necessity to vary

The interaction of bacteria with multicellular hosts represents a conflicting situation, the outcome of which is governed – under the permanent pressure of natural selection – by the tendencies of both interacting species to maximize their individual fitness. It is irrelevant whether the bacteria "aims" at living in the host or meets it rather coincidentally (coincidental evolution, see chapter B). From an ecological-evolutionary perspective, this "fight" for increasing the individual fitness can be likened to a genetic arms race which takes

simultaneously place at two different evolutionary time scales. Here we focus on events in bacterial populations assuming that the selection acting on host genes are relatively unimportant, especially in short-term because of the differences in generation times between the host and the bacteria.

Whereas it is a race between whole species, the bacterial species versus that of the host during their coevolution over long periods where microbial populations need to adapt to gross environmental changes encountered during the coevolution with the host, it is, in short-term, a race between the bacterial population infecting an individual host, and the immune system of that host. Both levels of coevolution have their own mechanisms to generate sufficient genetic variation to allow an adaptation through natural selection. In long-term, the bacterial population structures are the result of two genetic processes, (i) the genetic drift (mutagenesis) which is the consequence of different kinds of variation generators (mobile genetic elements, site-specific inversions, Arber, 1993 and 1995) occurring within the scope of a species, and (ii) horizontal genetic exchange affecting the species extrinsically. Thereby, population structures evolve ranging from rather clonal ones such as *Salmonella* spp. and *E. coli* which are mainly shaped by genetic drift, to panmictic populations with a high genome plasticity such as *N. gonorrhoeae* where horizontal genetic exchange is overwhelming and practically no genetic linkage is apparent (Maynard Smith et al., 1993; O'Rourke and Steven, 1994). As part of this short-sighted evolution, bacteria have to adapt to frequent, usually recurrent, microenvironmental changes during the course of infection. In order to respond to such recurrent changes, bacteria harbour intrinsic, retrievable genetic programs. The first program is gene regulation which influences the population as a whole. In response to a certain environmental stimulus, such as temperature, osmolarity, or specific substances (i.e. low iron concentrations), the bacteria alter the expression of responsive genes in a coordinated fashion (see Clark, 1990 for a review). The second program, genetic variation, antigenic and phase variation, concerns spontaneous changes in the DNA which are inherited to the progeny and are often reversible. Although such changes are temporarily random and take place at spatially distinct loci, they ultimately lead to the synthesis of altered gene products (antigenic variation) and/or the "on"/"off" switch of a particular protein (phase variation). Whereas gene regulation represents a well determined adaptive process for the benefit of the whole population, genetic variation programs produce heterogenous populations, minor parts of which may prove to be preadapted to virtually any unpredictable changes and may act as starting populations for subsequent clonal (vertical) outgrowth under a particular selectional constraint. These preadaptation processes to which programs for phase and antigenic variation contribute not only concerns counteraction to the host immune defence system but also generation of subpopulations which are

adapted to a particular of several microenvironments encountered during the course of infection. Although the course of infection of various pathogenic bacteria is as different as their pathogenic lifestyles, an often recurring theme of subsequently infected microenvironments (infection-stage associated transition) becomes evident (i) colonization of the epithelial cell layer, (ii) invasion of epithelial cells and (iii) persistence and dissemination in the blood and lymphatic system: systemic infection. In the following chapters we want to use this simplified three-step course of infection to describe strategies for antigenic and phase variation bacterial pathogens evolved in order to withstand the recurrent elimination effects by the hosts defence systems. Besides immune escape and infection-stage associated transitions, phase and antigenic variation is also a prerequisite for transmission to another host which has already been infected with the same pathogenic species and developed resistance.

2. *Genetic variation by genetic drift and horizontal exchange*

Genetic drift and horizontal exchange are two fundamental mechanisms which are thought to account for a steady background production of genetic diversity in microbial populations and guarantee under the permanent selectional forces of the environment the progress of evolution in the long-term (Arber, 1993 and 1995).

Whereas genetic drift affects a microbial population intrinsically with occasional clonal outgrowth of subpopulations upon selection, horizontal exchange can be considered as sharing successful genetic determinants with other populations. Besides their importance for long-term evolution, genetic drift and horizontal exchange also seem to be the origin of antigenic variation or antigenic drift respectively. Furthermore, horizontal genetic exchange of virulence factors via plasmid and phage across species may explain why for example different bacteria express a similar A-B type toxin or why *S. flexneri* and EIEC cause a similar disease with homologous proteins (see chapters B and D).

Although there must be an infinite number of examples for antigenic drift we want to mention two which both affect components on the bacterial cell surface: the major outer-membrane protein (MOMP P2; Murphy, 1994) of *H. influenzae* and the phase variation of the polysaccharide capsule of *P. aeruginosa* (DeVries and Ohman, 1994). Duim et al. (1994) showed that a *H. influenzae* population isolated from the respiratory tract of persistently infected patients show an antigenic drift in the surface-exposed loops of the molecule. The longest surface-oriented loops tend to be immunodominant and show the greatest variation among different strains (Haase et al., 1994; van der Ley et al., 1991). Obviously, these point mutations, which all produce amino acid changes, result from a selective advantage for the mutated bacteria under the pressure exerted by the

host immune system. A similar result with merely the same amino acid changes was shown by *in vitro* experiments in which bactericidal monoclonal antibodies specific for MOMP and complement were added to *H. influenzae* (Murphy, 1994; Duim et al., 1994).

In the other example, following colonization of the respiratory tract of cystic fibrosis patients with *P. aeruginosa*, mucoid variants of the original strain emerge and become predominant (Govan and Harris, 1986; Ogle et al., 1987). The mucoid phenotype is a result of the overproduction of the exopolysaccharide alginate (see chapter C1.) and confers an increased resistance to phagocytosis, thus allowing microcolony formation in the lung (Baltimore and Mitchell, 1982; Lam et al., 1980). However, the mucoid phenotype is not stable and reverts to a non-mucoid form *in vivo*. The switch causing this phase variation was found to lie in *algT*, a putative sigma factor involved in the alginate synthesis (Flynn and Ohman, 1988). The non-mucoid *P. aeruginosa* contain either of two missense mutations in *algT* occurring at hotspots (DeVries and Ohman, 1994). Revertants are recurrently selected upon infection of cystic fibrosis patients which are able to express *algT*. Given the fact that AlgT is a putative sigma factor, its production affects the expression of other virulence determinants such as proteases, exotoxin A and S, phospholipase C and the expression of the rough LPS all of which correlate with the mucoid phenotype (Luzar and Montie, 1985; Ohman and Chakrabarty, 1982; Wodds et al., 1991; Hancock et al., 1983).

Although horizontal genetic exchange, included transduction mobilization and natural transformation competence, seems to be of particular importance for bacterial pathogens since it allows the rapid horizontal spread of virtual any determinant of the whole gene pool and favours therefore the outgrowth of a microbial population – much faster than clonal outgrowth – which is optimally adapted to a particular microenvironment. Horizontal exchange may also be important in order to proceed with infection (infection-stage associated transitions) since it allows the rapid "collection" of traits needed to reach or survive the next microenvironment (Fussenegger et al., 1996a and b; Fussenegger et al., submitted).

All these reasons may explain why many pathogens are naturally transformation competent (Table 2). The importance for transformation-mediated horizontal exchange is particularly evident for *N. gonorrhoeae* which, based on the current knowledge, lacks any transducing phage or other mechanism which promote the mobilization of chromosomal determinants (Meyer et al., 1994). In fact, there exists ample circumstantial evidence for horizontal genetic exchange between commensal and pathogenic *Neisseria* spp., between meningococci and gonococci and within a pathogenic *Neisseria* species generating mosaic genes (Manning et al., 1991; Spratt et al., 1992; Feavers et al., 1992). Additionally, the exchange of virulence genes has been shown by co-

cultivation to occur within minutes with a frequency in the order of 10^{-5} per cell and genetic locus (Frosch and Meyer, 1992). However, not only variants of the *opa* gene family are transferred by horizontal exchange but also the loci encoding a particular structural pilin as one of several mechanisms for phase and antigenic pilin variation (Seifert et al., 1988; Gibbs et al., 1989, see 3.1.1.).

Table 2. Naturally competent human pathogens

Competent pathogens	Reference
Campylobacter jejuni	Wang and Taylor, 1990
Campylobacter coli	Wang and Taylor, 1990
Haemophilus influenzae	Mathis and Scocca, 1982
Haemophilus parainfluenzae	Gromkova and Goodgal, 1979
Helicobacter pylori	Haas et al., 1993
Moraxella species	Juni et al., 1988
Neisseria gonorrhoeae	Mathis and Scocca, 1982
Neisseria meningitidis	Catlin, 1960
Staphylococcus aureus	Rudin et al., 1974
Streptococcus pneumoniae	Lacks et al., 1975
Streptococcus sanguis	Behnke, 1981
Streptococcus mutans	Shah and Caufield, 1993

Given our simplified model that a course of infection consists of three steps (i) colonization, (ii) invasion and (iii) persistence in the bloodstream, a particular pathogen such as *N. gonorrhoeae* has to undergo typical alterations, infection-stage associated transitions, which render the bacteria optimally adapted to various microenvironments. Whereas the colonizing gonococcal phenotype expresses pili and long LPS, and heterogeneous Opa proteins, only bacteria which switch piliation off, express a distinct Opa (Opa_{30}) and produce short LPS can proceed with the infection and invade the epithelial cell layer. Furthermore, in order to become serum resistant, long LPS has to be expressed which is a prerequisite for its sialylation (see chapters C1. and 3.3.2.) and different pili and Opa proteins may be re-expressed depending on the site of persistence (Makino et al., 1991; Broome, 1986; van Putten, 1993). Although all of these switches can also be achieved by intrinsic mechanisms for phase and antigenic variation as described below (3.1.1.; 3.3.2.; 3.3.4.), the frequency of a rapid outgrowth of a population combining all traits of the respective colonizing, invasive or immuno-resistant phenotype, which is critical for the success of the infection or bacterial

persistence, greatly increases. By transformation-mediated horizontal genetic exchange, individual bacterial cells can rapidly acquire or collect the necessary determinant composition for the adaptation to a particular microenvironment from neighbouring bacteria before awaiting subsequent internal switches and clonal expansion. This could be the reason why components which are important for gonococcal adherence and invasion are also functionally involved in the DNA transformation process (Fussenegger et al., 1996b; Fussenegger et al., submitted; Rudel et al., 1995a).

Horizontal exchange and genetic drift represent a common repertoire for the generation of genetic diversity and is not limited to pathogens. In addition, these bacteria evolved several mechanisms for phase and antigenic variation - programmed genetic variation - which allow them to produce heterogeneous populations, a fraction of which may be preadapted to a particular microenvironment and to any future microenvironmental change.

3. Programmed genetic variation

3.1. Colonization of epithelial cells, genetic variation of motility and adherence

Colonization of the host is the first step of every infection and therefore of tremendous importance for its future outcome. Usually, bacteria reach the mucosal layer, either passively or by the help of flagella, where they may bind to the epithelial cells at first very loosely via pili or fimbriae followed by a more tight adherence mediated by a bacterial protein moiety and proteoglycan side chains on the cell surface of the target cell (with *L. monocytogenes* being the other way round). As a general rule, phase and antigenic variation can contribute by three different ways to the fitness of the pathogen: (i) surface exposed, immunogenic structures are varied or switched on and off in order to deceive the host immune system (immune escape), (ii) promote different functions, i.e. binding to different cell or tissue types (cell tropism) or (iii) allow or are important for transitions from one microenvironment to another (infection-stage associated transitions).

3.1.1. General homologous recombination (Fig. 2, 1.A, Table 1). Mechanisms of antigenic variation and to a lesser extent phase variation utilizing homologous recombination are widely distributed and mostly dependent on the RecA protein. Sequence analysis of pilin transcripts of the Gram-negative type 4 pili producing pathogen *N. gonorrhoeae* suggested that both phase and antigenic variation (reviewed by Meyer et al., 1990) might result from intragenic recombination events within the expression locus *pilE* (Meyer et al., 1982). It was further demonstrated that this recombinatorial exchange involved the transfer of variable minicassette sequences from a repertoire of more than 10 silent pilin

loci (*pilS*) - each of which encodes several independent variant pilin genes which are not transcribed and lack the invariant amino-terminal domain important for polymerization of the pilus - to one or two expression loci (Haas and Meyer, 1986; Swanson et al., 1986). Besides the recombinatorial integration of exogenous pilin information (silent or expression loci) taken up in a transformation-dependent manner (see chapter 2), intrachromosomal recombination or recombination between two sister chromosomes during DNA replication is manifested in either a reciprocal or a gene conversion-like exchange of the *pilE* gene in the expression locus (Seifert et al., 1988; Gibbs et al., 1989). Additionally, homologous recombinations between silent loci could further create new variable minicassettes or amplify the overall repertoire of silent, variable pilin genes. This is a variation mechanism which is generally found for gene families (see below, Opa protein variation). The reassortment of pilin sequences by recombination not only leads to the production of antigenically distinct pili, but it is also responsible for their phase variation. Besides sporadically occurring *pilE* deletion mutants which irreversibly lose their expression locus (or loci) (Bergström et al., 1986), revertible non-piliated phase variants fall into two groups. When propilin is cleaved at position +40 rather than at position +1 in a sequence-dependent manner, the hydrophobic, invariant N-terminus which is responsible for the polymerisation of the pilus fibre is lacking and the truncated pilus subunits are secreted into the medium (soluble pilin, S pilin, Haas et al., 1987). S pilin is thought to trap pilin-specific antibodies directed against a specific pilin type of a micropopulation which would lower the antibody response in the immediate surroundings of such a population. A second group of non-piliated variants produce very long L-pilin molecules which result from unequal recombination between *pilS* and *pilE* leading to the presence of multiple tandem in-frame copies of *pilS* minicassettes in the expression site (Manning et al., 1990; Gibbs et al., 1989). These very long L-pilin molecules are not assembly proficient and are found in the periplasm or the outer membrane of the bacteria thereby conferring elevated resistance to antibiotics. L-pilin variants can sometimes revert to the piliated phenotype when the tandem copy within the *pilE* gene is lost by a RecA-dependent deletion (Manning et al., 1990). Whereas the recombination between *pilS* and *pilE* loci enables a single *Neisseria* strain to produce theoretically 10^7 different variants, this number raises to near infinite if it is taken into consideration that, due to the presence of two expression loci, a gonococcal cell could express two different pili, a distinct pilus consisting of two variant pilin subunits or S-pilin combined with an intact pilus (Haas and Meyer, 1986; Meyer et al., 1984). Furthermore, pilin can be modified by phosphorylation and/or glycosylation (Virji et al., 1993b) and it has been recently shown that the variant-dependent glycosylation of gonococcal pilin can influence receptor recognition. It is therefore obvious that such a system challenges the variability

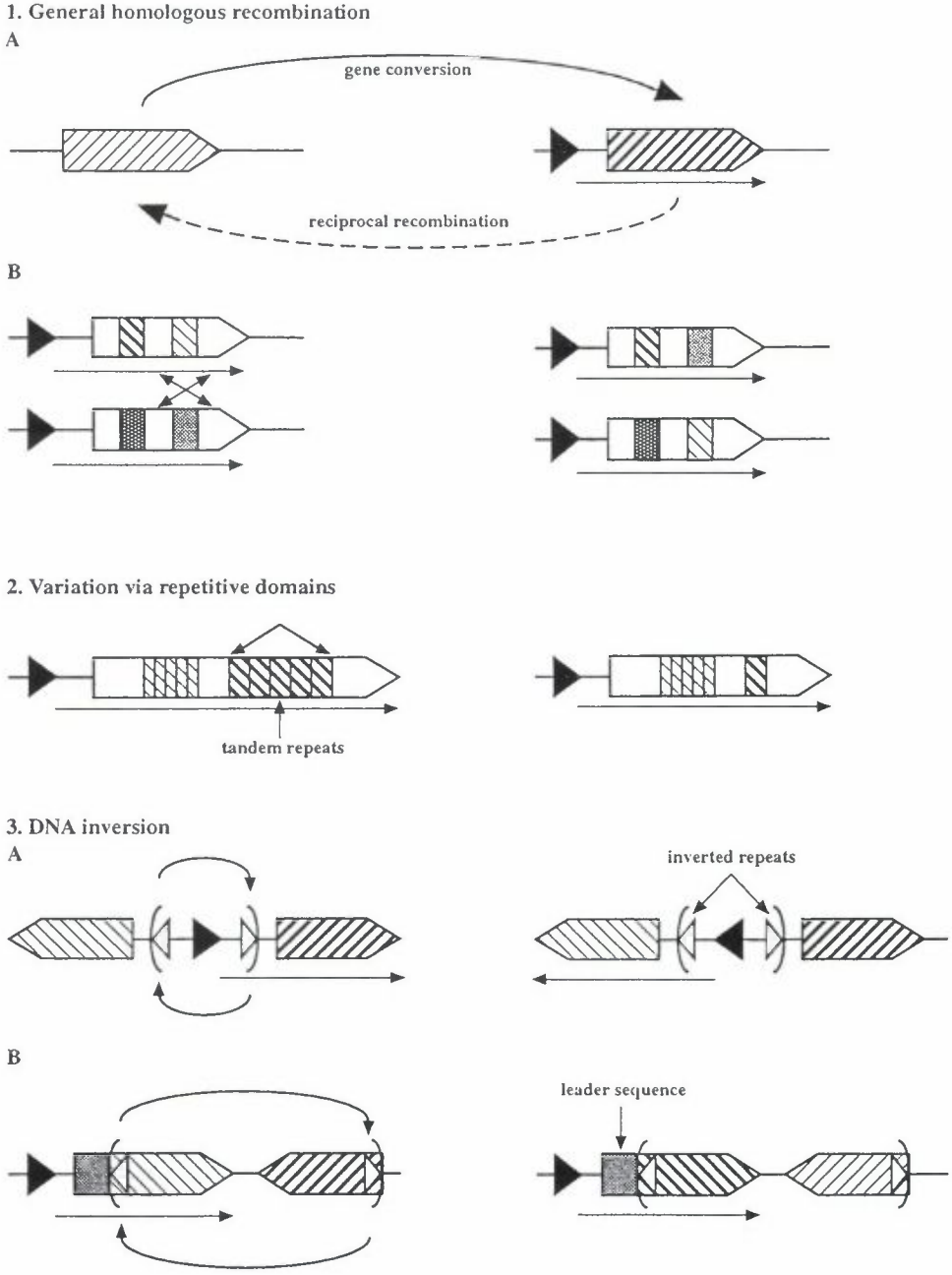
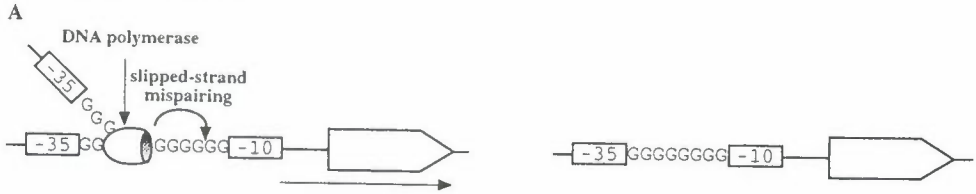
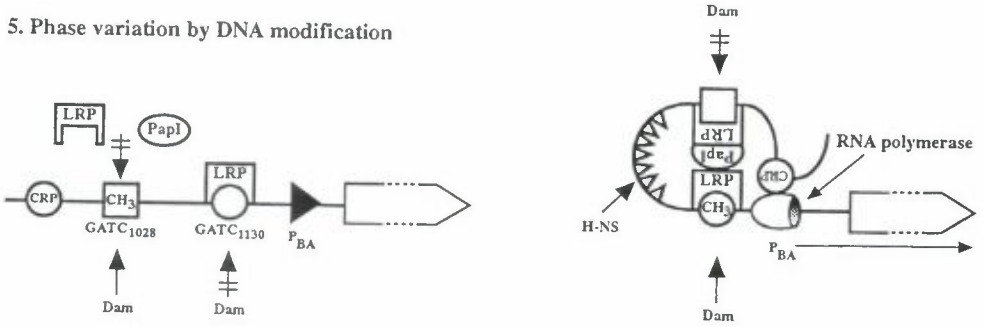


Figure 2. Different molecular mechanisms used by bacterial pathogens to produce phase and antigenic variation. For simplicity, homo- and heteropolymeric repeats shown in 3.A and 3.B are represented by polyG and polyC tracts, respectively. Examples for each variation type are shown in Table 1.

4. Variation via short repeats



5. Phase variation by DNA modification



6. Variation by mobile genetic elements

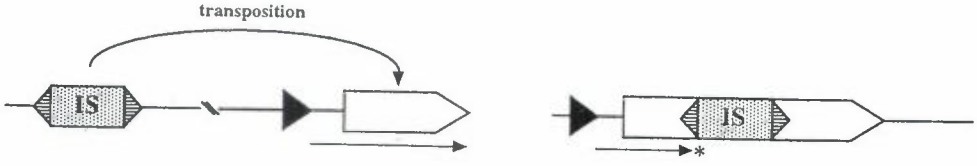


Figure 2. Continued.

mechanisms of the host immune system which is based on a similar mechanism recombining V, D, and J segments in B and T cells (Betz et al., 1993) and thus explains, why *N. gonorrhoeae* preserves its only natural habitat without being extinguished by the human immune system.

A similar mechanism as for gonococcal pilin variation namely homologous recombination of a silent gene into an expression locus has been evolved by *B. hermsii* and the protozoa *Trypanosoma brucei* in order to escape the immune system and become serum resistant (Donelson, 1995, see 3.3.1).

Following the initial, loose pilus-mediated contact to the host target cell, opacity proteins confer intimate binding and form a gene family of 11 independent copies on the gonococcal chromosome. Besides the independent "on" and "off" switch of individual genes described below (3.1.3.), RecA-dependent recombination events between the rather conserved parts of opacity genes is a source of variation leading to the reassortment of the hypervariable regions (Stern et al., 1986; Connell et al., 1988; Fig. 2, 1.B). Similarly, the insect pathogen *Bacillus thuringiensis* generates new toxin specificities active against different larvae species by recombination between and within a hypervariable region of the toxin genes which may exert the specificity of action. However, such recombinations were shown to occur independent of RecA (Caramori et al., 1991; Fig. 2.B, Table 1).

3.1.2. Site-specific inversion systems (Figs. 2, 3). Many pathogenic bacteria produce surface appendages involved in motility or attachment to surfaces and, since they are highly antigenic, they are often phase and antigenically variable. In contrast to the general recombination mechanism used by gonococci for pilin variation, other organisms use site-specific inversion of a genetic element to switch encoded fimbriae or flagella "on" and "off". The general inversion machinery consists of two inverted repeats flanking an invertible fragment, which contains depending on the respective system either a promoter (*E. coli* fimbrial expression, Abraham et al., 1985; *Salmonella* flagellar expression, Silverman et al., 1979; Figs. 2, 3.A) or two 3' termini of structural genes reading towards each other (pilin inversion of *Moraxella* spp., Marrs et al., 1988a and 1988b; Rozsa et al., 1991, phage tail fibre inversion systems, van de Putte et al., 1984, Kamp et al., 1978; Iida et al., 1982; Figs. 2, 3.B). A site-specific enzymatic reaction involves the transient binding of an invertase to both inverted repeats which is probably facilitated by DNA binding or bending proteins such as Fis (factor for inversion stimulation, Kahmann et al., 1985), IHF (integration host factor, Dorman and Higgins, 1987) and the histone-like protein HU (Johnson et al., 1986; Johnson and Simon, 1985). The co-operative binding of all factors leads to a specific parallel alignment of the DNA strands and the inverted repeats in a synaptic complex followed by transient cleavage of both DNA strands, rotation

of 180 degrees and religation (see Moskowitz et al., 1991, Heichman et al., 1991, and Johnson, 1991 for the mechanism). This leads to the inversion of the invertible DNA fragment thus placing either a promoter in front of another gene (*E. coli* fimbrial expression, *Salmonella* flagella expression; Fig. 2, 3.A) or fusing a constant 5' terminal part to another 3' variant part (pilin inversion of *Moraxella* spp., phage tail fibre inversion systems; Fig. 2, 3.B).

The Hin system of *S. typhimurium* controlling antigenic variation of flagellar antigens (H antigens) was serotypically discovered by R. W Andrewes in 1922. With a frequency of 10^{-3}– 10^{-5} these pathogens alternately express two different flagella which render the bacterium mobile but are also highly immunogenic. As a result of an immune response mounted against the flagella, the pathogen becomes immobile and is eventually killed. This constitutes a selectional advantage favouring or selecting those individuals in the population which, by taking advantage of the inversion system, had preadapted by expressing another of two possible flagella not recognised by the immune response mounted against the first one. The 996 bp invertible segment contains the site-specific recombinase gene *hin* and a promoter reading off the invertible segment. Upon inversion, the promoter is fused to a dicistronic reading frame containing the flagellar gene H2 and a repressor rH1 repressing the flagellar gene H1 which lies elsewhere on the chromosome. In the inverted orientation the promoter does not drive any gene which indirectly allows H1 expression as a consequence of the absence of its repressor rH1 (Silverman et al., 1979; Silverman and Simon, 1980).

A similar system with no homology to the Hin system has evolved in ETEC which produce type 1 fimbriae able to bind to mannosyl residues that are found in the mucus as well as on the host cells (Ofek et al., 1977). *E. coli* can switch their fimbrial expression "on" and "off" with a frequency of 10^{-3}. Abraham et al. (1985) reported that a cis acting element controlling the fimbrial phase variation was a 314 bp invertible DNA segment bounded by 9 bp inverted repeats which contains the promoter of the fimbrial subunit FimA. Unlike other site-specific inversion systems, the type 1 fimbrial inversion is exerted by two different components FimB and FimE encoded upstream of *fimA*. Whereas FimB promotes "on" inversion which fuses the promoter on the invertible segment to the *fimA* reading frame, FimE promotes "off" inversion. Owing to the high homology of the invertases FimB and FimE to the integrases of lambdoid phages, the inversion process is, as phage integration, dependent on IHF (Eisenstein et al., 1987). Flagellar phase and antigenic variation has also been reported to occur in *S. marcescens* and *Campylobacter coli* (Paruchuri and Harshey, 1987; Guerry et al., 1988). However, the mechanism of the corresponding DNA rearrangements have yet to be elucidated.

M. lacunata and *M. bovis* are the causative agents of human or bovine keraconjunctivitis respectively. Their pili are phase and antigenically variable

and display like those of *N. gonorrhoeae* a high affinity to corneal epithelial cells (Ringvold et al., 1985). Both *Moraxella* spp. can express two antigenically distinct pilin subunits due to an inversion event which determines whether I or Q pilin is expressed (Marrs et al., 1988a and b; Rozsa et al., 1991). The inversion appears to occur within the amino-terminal region of the pilin genes, and only the pilin gene which has the promoter and the 5' invariant portion of the gene produce pilin protein (Fig. 2 3.B). Thus, in one orientation of the 2 kb invertible DNA region the Q-pilin-specific sequences are in the expression locus adjacent to the promoter and the initial gene sequences which code for the leader sequence and the amino-terminal amino acids, while the I-pilin-specific sequences are in the inverted, non-expressed position. In the other orientation the reverse is true; that is the I-pilin-specific sequences are in the expression locus and the Q-pilin-specific sequences are in the inverted, non-expressed position. Besides the alternate expression of two antigenically distinct pili the inversion mechanism is also responsible for their phase variation. At least two different P^- variants arise with a frequency of 10^{-5} (i) phenotypically P^- bacteria that still produce original pilin protein and (ii) a second class of P^- isolates that produce no detectable pilin. This observation was explained by the occurrence of imperfect recombinational inversion events that lead either to a frame shift mutation or to an assembly missense polypeptide (Marrs et al., 1988a and b; Rozsa et al., 1991).

It is interesting to note that such closely related type 4 pili producing bacteria like *N. gonorrhoeae* and *M. lacunata* and *M. bovis* have evolved such different pilin variation mechanisms. However, we recently cloned a site-specific recombinase of *N. gonorrhoeae* (gonococcal recombinase, *gcr*) which is able to invert the *M. lacunata* pilin inversion system and are about to show the impact of Gcr on gonococcal pilin variation (Rozsa and Fussenegger, submitted). Similarly, site-specific invertases could be isolated from *S. aureus* and *Shigella* strains by functional complementation of the *Salmonella* Hin inversion system. The plasmid pI524 of *S. aureus* was reported to contain the invertible fragment *inv* which was proposed, due to its juxtaposition to an ampicilin resistance gene, to regulate antibiotic resistance upon inversion mediated by the Bin recombinase (Murphy and Novick, 1979; Rowland and Dyke, 1988). Three other site-specific invertases PinB, PinD and PinF were found in *Shigella* spp. but their role in pathogenesis as well as their target invertible fragments remain to be investigated (Tominaga et al., 1991).

An interesting variation mechanism combining homologous reciprocal recombination and inversion has recently been described in *Campylobacter fetus*, an animal pathogen (cattle, sheep and goats) causing abortions and sterility. This Gram-negative animal pathogen possesses an outermost crystalline surface layer of regular closely packed protein subunits, the S-layer proteins (SLPs). The *C. fetus* SLPs are a critical virulence factor in resistance to host immune defences

(Blaser and Pei, 1993). The antigenic variation of this surface structure takes place at two levels. There are six silent SLP encoding genes which could be activated upon reciprocal, possibly RecA-mediated, non-duplicative recombination into an expression locus. The expression locus consists of two divergent genes, *sapA* and *sapA2* which flank a 6.2 kb invertible DNA segment harbouring the promoter for alternate expression of *sapA* and *sapA2* (Dworkin and Blaser, 1996). The segment has been postulated to be inverted by site-specific recombination by an as yet unidentified invertase, but RecA-mediated recombination can not be excluded to date. Rearrangements involving a multi-cassette inversion process can also be considered, since each of the 8 SLPs (two in the expression locus and 6 silent loci) contains a conserved region that includes the putative inverted repeat (IR) upstream. DNA recombinases have been shown to invert chromosomal segments of over 200 kb in size (Rozsa et al., 1995) and 6.2 kb is easily possible (see inversion clusters below). This is further supported by the fact that unlike for the *pilE* system of *N. gonorrhoeae* (Gibbs et al., 1989), SLP recombination into the expression locus is exclusively reciprocal although homologous reciprocal recombination between two sister chromosomes would resolve in a gene conversion (duplicative recombination, see above) event detectable after cell division (Robertson and Meyer, 1992). Therefore, Dworkin and Blaser (1996) postulated that variation of the SLP expression locus involved a mechanism other than reciprocal recombination, probably a multi-cassette inversion process.

Besides their importance for bacterial pilin, flagellum or SLP variation, inversion systems are widely found on phages where they enable them to produce different tail fibres thereby determining the bacteriophage's host range. These inversion systems of phage Mu (Gin, Kamp et al., 1978), P1 (Cin, Iida et al., 1982) and e14 (Pin, van de Putte et al., 1984), a cryptic prophage, are highly homologous and functionally compatible with the Hin system with which they form the Din family of DNA inversion systems (Glasgow et al., 1989, for a review).

Two other systems, Min of P15B (a P1 derivative, Sandmeier et al., 1990) and the shufflon (*E. coli*, Komano et al. 1987) contain multiple tandem invertible segments which could produce variants in the range of 10^3. However, inversion clusters do not seem to be advantageous for an organism since no function could be attributed neither to the Min cluster nor the shufflon although the invertible Min fragments were shown to encode phage tail fibres (Sandmeier, 1994). The multiple inversion system may not be advantageous for producing genetic diversity since one particular determinant is rather unlikely to be expressed which results in the failure of the corresponding population to become large enough in order to withstand the selection by environmental factors as a whole.

3.1.3. Variation by homo- and heteropolymeric repeats (Fig. 4.B). Phase and antigenic variation based on homo- and heteropolymeric repeats is a peculiar expression control of genes which seems for most cases to be RecA-independent and relies entirely on the DNA polymerase which is prone to produce "errors" during DNA replication, especially when duplicating polymeric repeats.

This reproduction infidelity is thought to occur by slipped-strand mispairing (Meyer et al., 1987; Levinson and Gutman, 1987). Although replication infidelity has long been proposed to be a source of genetic diversity during long-term evolution (Arber, 1993 and 1995), polymeric repeats are somewhat hotspots of replication infidelity which reach a mutation rate of several percent per cell per generation. When the DNA polymerase finds itself during DNA replication in a polymeric repeat sequence which constitutes an identical sequence environment upstream and downstream of the DNA polymerase it could slip forward skipping repeats and then mispairs further downstream, thereby deleting repeats and shortening the overall repeat structure. In the other case, the DNA polymerase slips back thereby adding repeats to the repetitive structure (Fig. 3.). Based on *in vitro* experiments with the *opa* repeat region, Belland (1991) proposed that AT-rich regions which are able to form cruciforms or repeat structures which could form triple-stranded H-DNA may expose single-stranded DNA under negative superhelical tension. These single-stranded regions may be subject to nicking and subsequent partial degradation by exonucleases. Following degradation the gap would be repaired by DNA synthesis where mispairing occurs as described above, however, slipped-strand mispairing as proposed by Belland (1991) is independent of DNA replication.

There are two overall mechanisms by which slipped-strand mispairing results in phase and antigenic variation (Fig. 2, 4.). (i) The repeat structure lies within the promoter region flanked by the -10 and -35 boxes, and dependent on the repeat length, the promoter is either switched "on" or "off" or its strength is modulated. (ii) The phase control takes place at the translational level, where the length of the repeat, which is usually part of the signal sequence of a particular protein (coding repeat), determines the reading frame of the downstream translation, leading either to production of the corresponding protein or to a premature translational stop.

The opacity genes, a gene family of 11 genes of *N. gonorrhoeae* as well as the corresponding homologues in *N. meningitidis*, confer closer attachment to the host cell following initial pili-mediated binding and can independently be switched "on" and "off" due to several CTCTT pentamer units (from 8 to 28 coding repeats, Fig. 4.B) (Stern et al., 1986; Stern and Meyer, 1987). These pentameric repeats code for the hydrophobic core of the leader peptide without affecting the export function of the Opa protein. Considering the limited repertoire of variation by 11 genes, evasion of the host immune system is

probably not the main function. Opa variation may rather allow adaptive variation resulting in bacterial adhesion to various cells and tissues (cell tropism) since Opa proteins seem to able to bind to distinct cellular receptors, a capability, which is thought to lie within the two variable domains (Kupsch et al., 1993). However, this limited repertoire of a single cell can be altered or increased by recombination between different opacity genes as outlined above (chapter 2).

Yet another protein, PilC, was recently shown to be involved in adherence properties of *N. gonorrhoeae* and *N. meningitidis* (Rudel et al., 1995b; Nassif et al., 1994). A critical question emerges when describing the immune evasion mechanism of pili. How can they be infinitely variable and still at the same time allow functional integrity such as adherence to epithelial cells via a conserved receptor, and pilus polymerisation. The polymerisation function is non problematic because it involves the conserved hydrophobic regions of PilE which are neither surface-exposed nor immunosusceptible. The dilemma, however, is how to accommodate a conserved receptor binding function within the highly variable context of pilin. To solve the problem the bacteria make use of a minor, pilus-tip associated adhesin, PilC, which exists as two independent variant copies on the gonococcal and meningococcal chromosomes (Rudel et al., 1995b; Nassif et al., 1994; Jonsson et al., 1991). Both *pilC* genes can independently be switched "on" and "off" via a homopolymeric repeat consisting of polyG residues in their signal peptide-coding part (Fig. 4.B). However, PilC is not only localised at the pilus tip but also in the outer membrane where it acts as a component involved in pilus biogenesis and as such it can additionally become indirectly responsible for the phase variation of pili (Jonsson et al., 1991; 1992). Furthermore, in its function as pilus assembly factor it co-operates with PilE in the uptake of DNA during the transformation process of *N. gonorrhoeae* and is therefore also involved in the extensive horizontal genetic exchange seen with this pathogen (Rudel et al., 1995a; Fussenegger et al., submitted). Whereas both copies of PilC are functionally interchangeable with respect to epithelial cell adherence, pilus assembly and DNA uptake in *N. gonorrhoeae*, the situation is different in *N. meningitidis*. There, evidence exists also for a role of PilC proteins in meningococcal class I pilus biogenesis and in pilus-mediated adherence to epithelial and endothelial cells (Nassif et al., 1994), yet only one of the PilC proteins (PilC1) operates as an adhesin whereas the other (PilC2) does not (Nassif et al., 1994). The sequence and functional homologies of the *N. gonorrhoeae* and *N. meningitidis* PilC proteins suggest that they belong to the gene pool which is commonly used by both species via horizontal genetic exchange.

Besides gonococcal pili, type 1 fimbriae of ETEC and type 4 pili of *Moraxella* spp., three other pilus systems are subject to phase and antigenic variation, the Pap pili of uropathogenic *E. coli* (see 3.1.4., van der Woude et al., 1992) and the fimbriae of *H. influenzae* (van Ham et al., 1993) and *B. pertussis* (Willems et al.,

1990). It has long been known that the expression of *B. pertussis* virulence genes is affected by growth conditions such as temperature and the concentration of $MgSO_4$ (Clark, 1990 for a review). Under nonpermissive conditions, i.e. low temperature (25°C) or the presence of 20 mM $MgSO_4$ the virulence genes are repressed. When the cells are subsequently shifted to permissive conditions, production is resumed. Such a temperature and ion dependence of the expression of virulence genes is a quite common feature of pathogenic bacteria (i.e. *Yersinia* virulence genes) which can also live outside the human host. Upon mammalian infection, the increase in temperature to 37°C induces expression of virulence genes. Expression of these same virulence factors are disadvantageous during later steps of the infection which include dissemination and persistence in the bloodstream where they seem to be coordinately downregulated via i.e. the calcium concentration in the case of *Yersina* spp. (Clark, 1990). Recent evidence indicates that the response to temperature and $MgSO_4$ in *B. pertussis* is regulated by the *bvg* locus (*Bordetella* virulence genes). This locus encodes a two-component system with BvgS, a histidine kinase, that senses environmental signals and phosphorylates BvgA, the transcriptional activator of virulence genes. Under the control of the BvgS/BvgA system are virulence factors such as the two best studied adhesins Fha and the pertussis toxin, as well as the fimbriae (Stibitz et al., 1988; Weiss and Hewlett, 1986; Miller et al., 1989; Arico et al., 1989; Laoide and Ullmann, 1990). *In vivo, B. pertussis* undergoes frequent phase variation (10^{-6}) between a virulent state expressing the above mentioned genes and a nonvirulent state. This phase variation could be correlated with a frame shift mutation in a polyC tract in the *bvgS* gene (coding repeats, Fig. 4.B; Stibitz et al., 1988). Whereas the genes activated by BvgS/BvgA are determinants of adherence and initial colonization of tracheal tissue, they become superfluous of even deleterious to bacterial survival once the bacteria enter an intracellular phase via Fha-mediated, self-initiated phagocytosis by PMNs which avoids stimulation of the oxidative burst (Weiss and Hewlett, 1986). Therefore BvgS frame-shift mutants are selected which represent another example for infection-stage associated transitions.

B. pertussis produces two serologically distinct fimbriae, designated serotype 2 and 3 which are encoded by the corresponding *fim2* and *fim3* genes. *B. pertussis* strains contain a third fimbrial gene (*fimX*) which codes for an as yet unidentified product. In addition to the coordinate *bvg* locus-mediated regulation, fimbrial genes are subject to a second type of control, which occurs independently of other virulence genes. Thus, a particular *B. pertussis* strain may produce both types of fimbriae, only one type, or no fimbriae at all. These phase transitions occur by small deletions or insertions in a stretch of 15 C residues which lie between the -10 box and the binding site for an activator, a promoter configuration, which is commonly found in other positively regulated genes (Fig.

4.A). Thus, the length of the polyC stretch varied via slipped-strand mispairing influences the distance between the -10 box and the activator binding site (AB-site) thereby affecting the transcription of the *fim* genes (*fim2, fim3, fimX*) (Willems et al., 1990). The activator binding site is also present in promoters of other positively regulated genes which like the *fim* promoters do not contain a standard -35 region (Raibaud and Schwartz, 1984). Although the activator protein which binds to the AB-region has still not been identified, possible candidates are a *bvg* encoded polypeptide or another *bvg*-controlled gene which underlines the observation that Bvg regulation and phase variation of fimbriae are linked at the molecular level (Willems et al., 1990). Interestingly, the *fim* promoters show some homology with the pertussis toxin promoter (Nicosia and Rappuoli, 1987). It is significant that the pertussis toxin promoter contains 6 consecutive C residues at approximately the same relative position as the fim promoters. Indeed, when four of the six C residues are deleted, the *ptx* promoter is inactivated (Gross and Rappuoli, 1989) suggesting a similar role for the polyC stretch in the pertussis toxin and the *fim* promoters. It is an intriguing feature of *B. pertussis* toxin that it may act both as a toxin as well as an adhesin which is important for the first step during infection of this pathogen (Weiss and Hewlett, 1986; Saukkonen et al., 1992; see chapter B).

Another infection stage-transition has evolved in *H. influenzae*. During natural infection, nasopharyngeal isolates are often fimbriated while isogenic counterparts from systemic sites are as a rule nonfimbriated (Mason et al., 1985). The expression of *H. influenzae* fimbriae is variable and can have either of two levels of fimbriation or no expression (van Ham et al., 1993). These phenomena are controlled at the transcriptional level of two divergently oriented genes, *hifA* and *hifB*, encoding the major fimbrial subunit and the fimbrial chaperon, respectively. Whereas *hifA* is highly homologous to several types of *E. coli* fimbrial subunits (van Ham et al., 1989), *hifB* shows extensive homology to the chaperone gene of P pili. Therefore, the *hifB* gene product is a member of the family of periplasmic chaperon proteins (Holmgreen et al., 1992) which stabilise fimbrial subunits in an assembly-competent form (Kuehn et al., 1991). The *hifA* and *hifB* promoter regions were found to be clustered through an almost complete divergent overlap with a variable DNA backbone of repetitive TA units (van Ham et al., 1993). Variation in the number of TA units changes the normally strictly constrained spacing between the -35 and -10 sequences and controls the bi-directional transcription initiation, thus forming a mechanism directing multiple gene transcription (van Ham et al., 1993; Fig. 4.A).

3.1.4. Phase variation by DNA modification (Fig. 2, 5.). Genes encoding P pili are clustered on the chromosome of uropathogenic *E. coli*. These so-called Pap pili contain, similar to the gonococcal PilC, SfaS and FimH of the S pilus and type I

fimbriae of uropathogenic *E. coli* respectively (Lindberg et al., 1987; Moch et al., 1987; Abraham et al., 1988), a tip-associated adhesin PapG that binds globobiose on the host cell surface (Lund et al., 1987). In contrast to all other variation mechanisms, the P pilus phase variation does not include any DNA rearrangement and is entirely based on DNA modification. The promoter region (P_{BA}) of the *pap* cluster contains two methylation sites ($GATC_{1028}$, $GATC_{1130}$) to the A residue of which the Dam methylase adds methyl groups (Blyn et al., 1989; Blyn et al., 1990). The brief period of hemimethylation during DNA replication is used by bacterial repair systems to excise replication errors from the newly synthesised (unmethylated) strand and correct the error according to the template (methylated) strand (mismatch proofreading) (Modrich, 1987). Besides its importance for the mismatch proofreading system methylation can also protect against cleavage by restriction endonucleases (Arber and Linn, 1969). However, in the Pap pilus system, the methylation state also regulates binding of crucial activators for transcription of the *pap* gene cluster.

LRP (leucine responsive protein) binds to both methylated and unmethylated sites but only binding to methylated sites allows it to assume the conformation that activates RNA polymerase (Braaten et al., 1991; van der Woude et al., 1992). Thus, methylation of the site closest to the promoter of the *pap* gene cluster, P_{BA} ($GATC_{1130}$), is necessary for the binding of the active form of LRP. Furthermore, LRP can form a complex with PapI, a regulatory protein (Blyn et al., 1990). This complex only binds to unmethylated sites and if LRP-PapI binds to the $GATC_{1028}$ another prerequisite for the "on" configuration of the pap gene cluster is accomplished. If LRP and LRP-PapI bind to the appropriated sites they block subsequent Dam methylation, and if conditions in the cell are such that CRP (cAMP-binding protein) can bind its site, the active "on" complex can form and the pilin genes are transcribed. The involvement of LRP and CRP in the complex that binds to the operator region explains the response of pili expression to amino acid levels (LRP) and glucose levels (CRP) in the surroundings of the bacteria (van der Woude et al., 1992). The temperature regulation (see also above) may be mediated through H-NS, a histone-like protein, which supports "on" complexes by facilitating DNA bending (Fig. 2, 5.). An "on" to "off" switch is mediated by DNA replication which would dislodge LRP-PapI long enough for Dam methylase to gain access to the site (van der Woude et al., 1992).

Another variation phenomenon has recently been observed in the uropathogenic *E. coli* 536 (Blum et al., 1994). This pathogen carries two unstable DNA regions, pathogenic islands, which were shown to be responsible for virulence. These regions, 70 kb and 90 kb in size, contain the genes for hemolysin production (*hly*) and P-related fimbriae (*pfr*). By irreversible deletion between two direct repeats of 16 and 18 nucleotides, situated within two tRNA loci, *E. coli*

536 loses the pathogenic island of 70 kb or 90 kb respectively and concomitantly two important virulence genes (Blum et al., 1994).

3.2. Invasion of epithelial cells

Although proteins such as pili or fimbriae must be switched off during transition from the adherent to the invasive phenotype, there exist phase-variable proteins which require expression to allow host cell invasion and the progress of infection.

3.2.1. Variation by homo- and heteropolymeric repeats (Fig. 2, 4.). Opacity proteins were described above (chapters C1, 2, 3.1.3.) to confer intimate adherence of *N. gonorrhoeae* and *N. meningitidis* to epithelial cells but distinct Opa proteins of both species, OpaB of *N. meningitidis* (Virji et al., 1993a) and Opa_{30} of *N. gonorrhoeae* (Makino et al., 1991) need to be expressed in order to allow invasion of host cells. In *N. meningitidis*, the Opc protein, although structurally unrelated to Opas, has recently been described to have a function similar to the epithelial cell-specific Opa proteins (Virji et al., 1992; 1994). Like opacity proteins, Opc shows a rapid variation within a bacterial population. However, whereas Opa protein expression is regulated at the translational level (Stern et al., 1986, see above), regulation of Opc expression takes primarily place at the transcriptional level and is mediated by the length of a polycytidine stretch in the promoter region (Sarkari et al., 1994, Fig. 2, 4.A). Besides mediating invasion of epithelial cells, Opc is also involved in the invasion of endothelial cells (Virji et al., 1992; 1993a; 1994). Since Opc is immunogenic in humans and stimulates bactericidal antibodies (Rosenquist et al., 1993), *opc* expression should be switched "off" again during later stages of the infection process such as the persistence in the bloodstream (infection-stage associated transition). As described above, the width of transcriptional level can vary from no expression to very strong expression and is dependent on which spatial configuration -10 and -35 promoter sequences take with a particular homopolymeric repeat length (Sarkari et al., 1994).

The genus *Yersinia* provides some clues to the transition that pathogens can make between commensalism and virulence. Both, *yopA* encoding an outer membrane protein (Bolin et al., 1982) and the *inv* gene encoding the invasin (Isberg and Falkow, 1985) are present on the chromosomes of *Y. pseudotuberculosis* and its highly virulent relative *Y. pestis*. However, both *yopA* and *inv* are only expressed in *Y. pseudotuberculosis*. Although the overall *Y. pestis yopA* sequence differs only slightly from that of *Y. pseudotuberculosis* it harbours a single base deletion in a polyA tract (coding repeat) which shifts it out of frame (Rosqvist et al., 1988). Restoration of *Y. pestis* to $YopA^+$ reduces its virulence, and the introduction of mutations into the *Y. pseudotuberculosis yopA* and *inv*

genes greatly increases its virulence. Thus, mutations in two genes of *Y. pseudotuberculosis* could cause it to become highly virulent one of which, YopA can probably be phase varied by slipped strand mispairing (Fig. 2, 4.B). While many other pathogens tend to become less virulent as they adapt to their host, ultimately becoming commensal, these *Yersinia* spp. have maintained the potential to switch between virulence states, perhaps because this aids in transmission of the organism, while otherwise maintaining the population in endemic hosts at lower virulence. These results could provide an explanation for the observation of strains of *Y. pestis* of lower virulence and also for the sudden appearance of plague epidemics. The endemic host of *Y. pestis* may have harboured a strain of lower virulence, which by one mutation could have become hypervirulent. The reverse mechanism would also explain the decline and termination of outbreaks.

3.3. Persistence in the blood stream, evasion of the host immune system

3.3.1. General homologous recombination (Fig. 2). Evasion of the mammalian immune response by periodically changing the molecular constituents on the bacterial cell surface is one of the major reasons for the phenomenon of antigenic variation. Two pathogens particularly adept at using this strategy are *B. hermsii*, a prokaryotic spirochete that causes relapsing fever (Barbour and Hayes, 1986) and African trypanosomes, eukaryotic protozoan parasites that cause sleeping disease in Africa (Donelson and Rice-Ficht, 1985). *B. hermsii* is transmitted by ticks and African trypanosomes by tsetse flies. Both of these organisms circulate extracellularly in the bloodstream of their mammalian hosts and keep one step ahead of their host's immune systems by periodically switching the major protein of their surface. Although trypanosomes are not prokaryotic pathogens they should be included here since their antigenic variation mechanism resembles the one of *B. hermsii*.

Frequent evasion of the immune system by *B. hermsii* results in infections that relapse at regular intervals which are so characteristic that this disease is known as relapsing fever. The pathogenesis of the sequential relapses is unique: the organisms of each successive attack show antigenic differences, and circulating antibodies specific for the organisms of each onset appear in the blood which extinguish a particular population. The next relapse depends on the outgrowth of antigenically distinct mutants, against which the host then elaborates new antibodies. At least 40 antigenically distinct serotypes can arise from a single cell of *B. hermsii* each of which appears to be due to the expression of a different outer membrane lipoprotein, called the variable major protein (Vmp) (Plasterk et al., 1985). All *vmp* genes studied to date are located on linear plasmids (Plasterk et al., 1985; Kitten and Barbour, 1990). In a given bacterium all but one *vmp* genes are transcriptionally silent. A typical Vmp alteration is exemplified by the switch

from *B. hermsii* serotype 7 to serotype 21 (Plasterk et al., 1985; Kitten and Barbour, 1990). The silent *vmp21* on one linear plasmid is duplicated, and the duplicated copy is transposed downstream of a promoter at a telomere-linked expression site on another linear plasmid (Plasterk et al., 1985; Kitten and Barbour, 1990). This duplicative translocation is equivalent to a gene conversion event involving the unidirectional, nonreciprocal transfer of nucleotide sequences to a new site from a donor gene that remains unchanged after the transfer (Fink and Petes, 1984). The boundaries of the duplicated regions usually are a short common sequence just upstream of the start codons of silent and expressed genes and a 200 bp conserved sequence located downstream of their stop codons (Kitten and Barbour, 1990; Barbour et al, 1991). A second variation mechanism is associated with a frequently detected switch from serotype 7 to serotype 26 (Restrepo et al., 1994). In this case, a pseudogene version of *vmp26*, called *yvmp26*, is located immediately downstream of *vmp7*, both in the telomere-linked expression site of one linear plasmid, and in another linear plasmid containing the correspondingly linked donor genes. In the switch from *vmp7* to *vmp26* 20 bp homologous regions common to both genes serve as sites for recombination which fuses *vmp26* to the 5' end of *vmp7* which is subsequently deleted. Thus, an intramolecular deletion juxtaposes coding regions derived from two genes and generates a functional composite gene, an event reminiscent of DNA rearrangements associated with the creation of vertebrate immunoglobulins. Briefly, individual genes responsible for the primary antibody repertoire are assembled by intrachromosomal DNA rearrangements that juxtapose a member of the variable region (V) genes adjacent to a member of the diversity (D) and/or joining (J) elements and a representative of the constant region gene family. The diversity of this repertoire is enhanced by the multiplicity of V genes and the D and J elements (combinatorial diversity) and imprecision in the duplex DNA joining process (junctional diversity) (Betz et al., 1993). Another mechanism which is imposed on top of the above mentioned variation can generate additional variation by post-switch mutations of the *vmp* gene in the expression locus. Thereby partial gene conversions appear to occur that are templated from *vmp* pseudogenes located on the same linear plasmid as the expression site (Donelson, 1995).

Antigenic variation of the variant surface protein (VSG), a glycolipid-anchored glycoprotein with which trypanosomes are coated, has several features described above for the *B. hermsii* Vmps but with an apparent increase in complexity. Within the trypanosome genome there are as many as 10^3 different *vsg* genes (Van der Ploeg et al., 1982) some of which are closely related isogenes but only one is usually expressed at a time. The transcriptionally silent *vsg* genes are scattered on various chromosomes, with up to 20 different potential expression sites situated near a telomere (Clayton, 1988). The activation of a new *vsg* and

formation of a new serotype are often associated with one of three types of rearrangements: (i) Duplicative transposition (gene conversion) of a silent, donor *vsg* to a telomeric expression site, displacing the existing *vsg* (Hoeijmakers et al., 1980) by homologous recombination between 5' repeat sequences and 3' coding regions of *vsg*. (ii) Telomere conversion is a variation of duplicative transposition (i) whereby one entire telomeric region including its expressed *vsg* is replaced with a duplicated copy of another telomeric region and its silent *vsg*, followed by activation of the duplicated silent gene (Bernards et al., 1984). (iii) Reciprocal telomere exchange activates some *vsg* genes and inactivates others (Pays et al., 1985). A dramatic outcome of some *vsg* gene conversions is the formation of mosaic genes which seem to be a common theme of gene families as described above for the opacity genes of *N. gonorrhoeae* (chapter 2). In some cases, the newly created *vsg* is generated during the duplication event via multiple crossovers among related donor pseudogenes and can be built up from up to three closely related donor genes (Kamper and Barbet, 1992). Still other *vsgs* that are already telomere-linked can be activated *in situ* without apparent DNA rearrangement. It has been suggested for both *B. hermsii* (Restrepo and Barbour, 1994) and African trypanosomes (Gommers-Ampt et al., 1993) that nucleotide modification might be involved in regulating the expression of the *vmp* or *vsg* once it has reached its respective telomere-linked expression site. *B. hermsii* has a *dam* methylation system (Hughes and Johnson, 1990) that could possibly methylate silent *vmp* genes (Restrepo and Barbour, 1994) – a system reminiscent of the Pap pilus phase variation – whereas trypanosomes have an unusual glycosylated hydroxymethyluracil at some positions within silent telomere-linked *vsg* genes (Gommers-Ampt et al., 1993). However, proof that these modifications in either *B. hermsii* or trypanosomes regulate gene expression remains to be provided.

3.3.2. Variation by homo- and heteropolymeric repeats (Fig. 2, 4.). The major outer membrane proteins of *N. meningitidis* are of interest, since they are responsible for the serological differentiation of strains, and are therefore under investigation as components of experimental vaccines against meningococcal infection. The class 1 protein is a pore-forming protein with cationic selectivity (Tommassen et al., 1990) and is the product of the *porA* gene locus (Barlow et al., 1989). PorA has, for several years, been a potential vaccine candidate since immunization experiments in mice with outer membrane complexes produces bactericidal antibodies directed mainly against class 1 outer membrane proteins. Monoclonal antibodies against class 1 protein are bactericidal and confer protection in an animal model (Saukonen et al., 1987). PorA is expressed by most of the clinical isolates but with variation in the levels of expression. However, there were clinical isolates described which lack this class 1 protein despite the presence of a

chromosomally encoded *porA*. These PorA-negative phase variants had no *porA* transcript. Sequence analysis of the promoter region showed a polyG stretch flanked by -35 and -10 domains (Van der Ende et al., 1995; Fig. 2, 4.A). The length of the polyG stretch varies and is associated with the expression level of the class 1 outer membrane protein. Thus the transcription of the *porA* gene is likely to be regulated by changes in the polyG stretch length which presumably arises from slipped-strand mispairing of this region during replication (Van der Ende et al., 1995). Phase variation of class 1 outer membrane protein is a possible mechanism to evade the host immune defense. Therefore, the protective efficacy of a vaccine based on class 1 outer membrane protein may be questioned. A homologous *porA* has also been found in *N. gonorrhoeae* which also shows a polyG stretch in the promoter region (Andreas F. Kahrs, unpublished).

In *H. influenzae*, Kimura and Hansen (1986) described spontaneous, high-frequency, reversible acquisition and loss of reactivity with oligosaccharide-specific monoclonal antibodies and that these phenotypic changes were associated with susceptibility to serum killing and virulence for infant rats. The frequency with which loss or gain of phase-variable epitopes occurs is usually about 10^{-2} per bacterium per generation and the "on"/"off" switch can involve both independent and co-ordinate switching of epitopes (Weiser et al., 1989). Similar phase-variable switching of LPS epitopes has been observed in *N. meningitidis* and *N. gonorrhoeae* and their closest commensal relative *N. lactamica* (Virji et al., 1990; van Putten, 1993), but apparently does not occur in the enterobacteriaceae although LPS with variable lengths of O antigens play an important role in the pathogenesis of *Salmonella* spp. (variation mechanism not yet elucidated, see C3). LPS usually plays a role at two distinct steps during the infection process. Short LPS is needed for the invasion of host cells which appears more related to surface charges and steric hindrance of the invasion process rather than due to a specific function of LPS. Long LPS mediates serum resistance sometimes with additional sialylation as in the case of *N. gonorrhoeae* and *N. meningitidis* (van Putten, 1993; Hammerschmidt et al., 1996). Three loci control LPS variation in *H. influenzae*, *lic1A*, *lic2A* (or lex-1, Cope et al., 1991) and *lic3A* among which *lic1A* is the best characterised (Weiser et al., 1989; Maskell et al., 1991; High et al., 1993). *lic1A* contains in its 5' coding region immediately downstream of 3 ATG (start) codons a polytetrameric repeat sequence CAAT the length of which seems to vary from 29 to 31 by the mechanism described above: slipped-strand mispairing. Through loss or gain of CAAT repeats, frame shifts may occur with respect to upstream initiation codons resulting in altered translation. Two of the three possible frames could initiate translation, with the first frame containing two ATGs and the second containing the third ATG (Weiser et al., 1989). Unlike "on" or "off" phase variation by coding repeats in *N. gonorrhoeae* (*opa*, *pilC*), three levels of expression of the phase-variable LPS

epitopes in *lic1A* were observed in *H. influenzae*: strong, weak and undetectable. These different levels of expression could be correlated with the three possible reading frames. Recently, a new tetrameric repeat unit, GCAA, was found to be associated with the LPS biosynthetic genes of the *lex2A* locus (Jarosik and Hansen, 1994). Another phase variation which was first attributed to altered LPS (Weiser et al., 1995) is phenotypically evident during the course of *H. influenzae* infection as distinctive transparent colonies (T) representing the colonization phenotype of the nasopharynx. After going through an intermediate state (I) they become opaque and more resistant to serum killing as *H. influenzae* develops an invasive infection. Although this phenotypic switching was proposed to lie in the independent phase variation of *oap*, a novel gene required for expression of a membrane protein which mediates efficient nasopharynx colonization and the capsule production, the molecular mechanism of phase variation of both *oap* and capsule genotypes needs yet to be elucidated (Weiser et al., 1995; Moxon et al., 1996). Similarly, *S. pneumoniae* undergoes spontaneous phase variation in colony morphology. Differences in colony opacity have been shown to correlate with differences in the ability of these organisms to colonize the mucosal surface of the nasopharynx in an animal model (Cundell et al., 1995). Recently, intergenic, repetitive elements were shown to be responsible for the phase variation, possibly by altering expression of a putative regulatory gene downstream from the box elements (Saluja and Weiser, 1995). However, the exact molecular mechanism which is responsible for the phase variation as well as the gene(s) involved have yet to be described.

The sequencing of the entire chromosome of *H. influenzae* revealed four other tetrameric repeat motifs: CAAC, GACA, AGTC, and TTTA (Fleischman et al., 1995). Eleven loci were found to have multiple tetrameric repeats located within open reading frames. All of them show homology to virulence-associated genes from pathogenic bacteria such as a homologue of the *Yersinia* adhesin *yadA* associated with GCAA repeats and several homologues of neisserial transferrin binding proteins associated with CAAC repeats (Hood et al., submitted; Peak et al., 1996). Such a tranferrin binding protein homologue was also found in *Moraxella catarrhalis* which appears to be associated with the CAAC repeat (Peak et al., 1996).

Recently, a locus involved in the biosynthesis of gonococcal LPS has been cloned and was shown to contain five genes, *lgtA*, *lgtB*, *lgtC*, *lgtD* and *lgtE*, all of which code for different LPS glycosyltransferases, enzymes involved in the build up of the sugar structure of LPS (Gotschlich, 1994). *lgtA* and *lgtB* are highly homologous to *lgtD* and *lgtE* whereas *lgtC* shows homology to the *E. coli rfaI* and *rfaJ* genes involved in the LPS core synthesis. *lgtB* and *lgtE* show strong homology to the *lic2A* gene of *H. influenzae*, but do not contain the CAAT repeat found in this gene (Cope et al., 1991; High et al., 1993). However, the DNA

sequence analysis revealed that *lgtA*, *lgtC* and *lgtD* contain polyG tracts of 17, 10, and 11 bp respectively (coding repeats) (Gotschlich et al., 1994). Thus, three of the LPS biosynthetic enzymes of *N. gonorrhoeae* are potentially susceptible to premature termination by reading frame changes. It is likely that these structural features are responsible for the high-frequency genetic variation of the gonococcal LPS which has long been described (Schneider et al., 1988). A major difference among the variant LPS molecules is the presence of additional carbohydrate residues in the longer LPS forms that can be externally modified by membrane-associated bacterial sialyltransferase using host-derived or endogenous CMP-NANA as sialyl donor (Mandrell and Apicella, 1993; van Putten, 1993). Only sialated LPS mimics host cell surface structures preventing host immune attacks (van Putten 1993). Recently, a similar locus which encodes three genes of putative LPS glycosyltransferases has been cloned in *N. meningitidis* based on their homology to *lic2A* of *H. influenzae* (Jennings et al., 1995). The first open reading frame, *lgtA*, was found to control the phase-variable expression of the LPS terminal lacto-*N*-neotetraose via slipped-strand mispairing based variation of a homopolymeric tract of 14 guanosine residues in the coding sequence (Jennings et al., 1995). Additional genes of *N. meningitidis* and *N. gonorrhoeae* varied via polymeric repeats await further characterization. Peak et al. (1996) identified multiple GCAA repeats on the chromosomes of both pathogens, and Jennings et al. (1996) identified a gene in *N. meningitidis* with high homology to *icsA* of *S. flexneri* (Lett et al., 1989) which harbours this same coding repeat (GCAA).

3.3.3. Variation via repetitive domains (Fig. 2, 2.). Another variation theme involves the use of repetitive domains which differs from the variation used for the *B. hermsii* surface proteins (Vmp) and the gonococcal pilus protein (PilE) in only one way: In the antigenic variation involving repetitive domains the partial homologies that participate in the recombination event are intragenic instead of intergenic.

The Gram-positive group A streptococcus, *S. pyogenes*, would be susceptible to clearance by the immune system of the host except for the presence on its surface of a fibrous layer of M protein, the primary virulence factor of this bacterium (Lancefield, 1962; Fischetti, 1991) that consists of extended α-helical coiled-coil dimers (Fischetti et al., 1988). However, opsonization of the bacteria by antibodies directed against M protein would allow it to be phagocytosed and destroyed. Consequently, M protein is highly variable in size with over 80 antigenically different serotypes identified so far (Fischetti et al., 1985). The serotype M6 has been studied in detail and amino acid sequence analysis revealed the mechanism underlying the observed polymorphisms. The most striking structural feature of the M6 protein is the presence of extensive

reiterated segments (Hollingshead et al., 1986). Region 1 consists of five tandem repeats of 42 bases each ($A_0 - A_4$), and each of these is composed of two almost identical 21 bp repeats. Region 2 contains five tandem direct 75 bp repeats ($B_0 - B_4$) and region 3 contains two direct repeats of about 81 bp (C_1 and C_2) (Hollingshead et al., 1987). In these repeats, the external blocks (i.e. A_0 and A_4 or B_0 and B_4) diverge slightly from the consensus whereas the inner ones are identical (A_1, A_2 and A_3, B_1, B_2 and B_3). Analysis of the sequence of variant M proteins indicated that all mutations appear to occur by homologous recombination among reiterated DNA blocks within the gene, thereby generating both duplications as well as deletions (Hollingshead et al., 1987). However, it is still a matter of debate whether these recombinational events are mediated by a functional recombination system (RecA) or occur by replicative "slippage" as often described above, but the final result is indistinguishable. There are three main recombination events which lead to (i) the lack of production of M protein by creating a frame shift upon improper recombination, (ii) the shortening or lengthening of the protein mediated by duplication or deletion of identical repeats and (iii) the creation of novel domains (Hollingshead et al., 1987). Recombinations between identical repeat blocks, i.e. between A1 and A3 shorten the protein without neither altering the amino acid sequence nor the coiled-coil quaternary structure of the molecule since A blocks contain 14 amino acids (7 in each sub-block), and the appearance of a hydrophobic amino acid in every first and fourth place in a seven-residue sequence is a prerequisite for the coiled-coil structure. However, recombination between repeats A_0 and A_4 which are slightly different would create besides a shortage of the overall protein the creation of a new epitope. The same is the case for the B repeats but recombinations between these repeats could furthermore alter the coiled-coil structure of the dimeric molecule since B repeats contain 25 amino acids which is not an integral multiple of seven (Hollingshead et al., 1987). Besides the phase and antigenic variation based on DNA rearrangements, reversible phase switches between M^+ to M^- phenotypes were observed and this switching was associated with the corresponding change in colony opacity from Op^+ to Op^- as well as with the expression of the streptococcal C5a peptidase (Scp, Simpson et al., 1990). The locus controlling all three virulence determinants designated VirR (virulence regulator) encodes a trans-acting product that activates the expression of a number of distinct *S. pyogenes* genes (Simpson et al., 1990). One protein encoded by the VirR locus is homologous to effector proteins of two component sensor/effector regulator systems such as already described above for *B. pertussis* (Perez-Casal et al., 1991; 3.1.3.).

Two other examples of variation via repetitive domains should shortly be mentioned although they are not human pathogens but belong to species which also harbour human pathogens. The rickettsia *Anaplasma marginale* which causes

a hemoparasitic disease of cattle shows a size polymorphism of its major surface protein 1 (Msp1) (Allred et al., 1990). This protein displays overall homology between isolates but contains a domain with various numbers of tandemly repeated sequence, the number of which correlates with the size of the surface antigen. However, there is no correlation with antigenic variation in this region. Variation in repeat numbers probably occurs by slipped-strand mispairing or by homologous recombination (Allred et al., 1990).

Mycoplasma represent a group of about 80 diverse prokaryotic species most of which are parasites in animals or man. The antigenic diversity generated by the wall-less swine pathogen *Mycoplasma hyorhinis* is particularly intriguing since it combines several variation mechanisms mentioned above. The unusual structural basis for the variation of the membrane surface lipoproteins (Vlps) was revealed in a cluster of related but divergent *vlp* genes, *vlpA*, *vlpB* and *vlpC*, which occur as single chromosomal copies (Yogev et al., 1991). These encode N-terminal domains for membrane insertion and lipoprotein processing, but divergent external domains undergoing size variation by loss or gain of repetitive intragenic coding sequences while retaining a motif with distinctive charge distribution. Each of the three Vlps shows phase variation owing to highly conserved *vlp* promoter regions which contain a tract of contiguous A residues immediately upstream of the -10 box and is subject to frequent mutation altering its length in exact correspondence with the "on" and "off" phase states of specific genes. Thus, random combinatorial expression of these products, superimposed with independent size variation yields over 10^4 structural permutations of Vlps (Yogev et al., 1991).

A particularly striking feature found in the vast majority of wall-associated proteins is that they harbour tandemly repeated sequences either of one type or several distinct types, and in some cases a very large proportion of the molecule can consist of repeated sequences. The initial production of such repeats is still not clearly understood although one could speculate that, like for the variation of the M protein, it results from slippages during replication or unequal intragenic recombination events thereby producing at least two repeats which can then be subject to further variation. Unlike for the examples mentioned above, the advantages of possessing apparently unstable tandem repeats is not clear at present. It is possible that the ability to reversibly shorten or lengthen a cell-surface protein might facilitate an organism's ability to adapt to changing environmental conditions. Tandem repeats in many proteins have been associated with binding domains for other proteins or polysaccharides (Wren, 1991) and an ability to reversibly alter the number of such binding domains might also be important in adapting to changes in the environment. Taken together the recombination between tandem repeats may be seen as a general mechanism of bacterial surface proteins to accelerate sequence diversity.

Interestingly, the number of repeated sequences is mostly divisible by three which alleviates frame-shift mutations. There are many examples for cell wall-associated virulence factors which harbour tandem repeat sequences. Immunoglobulin Fc-binding proteins such as protein A (IgG binding, *S. aureus*, Patel et al., 1987), protein G (IgG-binding, *S. pyogenes*, Bjorck and Kronvall, 1984; Filpula et al., 1987; Fahnestock et al., 1986) as well as the M-like proteins Mrp (IgG-binding, Boyle et al., 1994) and Enn (IgA-binding, Bessen and Fischetti, 1992) of *S. pyogenes* bind the constant part of particular immunoglobulins, thus coating the bacteria in a way that does not lead to opsonization and may also prevent recognition by the host immune system. Recently, Mrp has also been shown to contribute to group A streptococcal resistance to phagocytosis by human granulocytes (Podbielski et al., 1996). Other proteins of the tandem repeat type display adhesive properties such as the *S. aureus* proteins Clf, the clumping factor mediating binding of the pathogens to fibronectin (Kehoe et al., 1994; McDevitt et al., 1992; Phonimdaeng et al., 1990), the collagen adhesin Cna which allows the colonization of collagen-rich tissues such as bone or cartillage or the fibronectin binding proteins of *S. aureus* (Patti et al., 1992; Flock et al., 1987) and *S. pyogenes* (Hanski and Caparon, 1992) which are involved in heart valve endocarditis (Kuypers and Proctor, 1989). The coagulase of *S. aureus* indirectly triggers fibrin polymerization and causes the fibrin material to be deposited on the cocci which may protect them from attack by host cells. Coagulase antagonizes the action of staphylokinase mentioned above (Boden and Flock, 1989; chapter A). PspA, a major outer surface protein of *S. pneumoniae*, appears to play a role in virulence and shows like M proteins both size variation and antigenic variation. However, unlike M protein, distinct serotypes are cross-protective indicating conservation of accessible protective epitopes (Crain et al., 1990). Although tandem repeat containing surface exposed proteins are mainly found in Gram-positive pathogens, a virulence-associated lipoprotein of *N. gonorrhoeae*, H8, has been shown to contain 14 tandemly repeated pentapeptides (Baehr et al., 1989). Most of the tandem repeat containing proteins mentioned above are clearly involved in bacterial virulence, yet their putative phase and antigenic variation due to those repeat units as described for the M protein is either absent (H8 proteins), irrelevant to immune evasion (PspA) or await further characterization. Therefore only M protein is included in Table 1. However, as a general mechanism of antigenic variation, intergenic recombination as described for the Opa gene family of *N. gonorrhoeae*, has also been reported to account for the domain shuffling observed for M-like and M proteins (Whatmore and Kehoe, unpublished).

3.3.4. Variation by mobile genetic elements. The polysaccharide capsule of *N. meningitidis* which consists of homopolymers of sialic acids, undergoes infection

stage dependent phase variation (Hammerschmidt et al., 1996). Sialic acid is also found as a modification of the meningococcal LPS (Mandrell, 1993) which is thought to undergo a similar phase variation as the one of *N. gonorrhoeae* (see 3.3.2.). The meningococcal capsule has to be switched "off" to allow Opa- and Opc-mediated invasion of the host cell, but later infection stages favor capsule production since it confers resistance to serum killing (Stephens et al., 1993; Virji et al., 1993a; Hammerschmidt et al., 1994; Jarvis, 1995). This capsular phase variation correlates with the endogenous sialylation of LPS. The underlying mechanism involves a reversible insertional inactivation of the *siaA* gene essentially involved in the synthesis of CMP-NeuNAc, which is required for both, capsular polysaccharide synthesis and endogenous LPS sialylation (Hammerschmidt et al., 1996). The mechanism of reversible inactivation of the meningococcal *siaA* gene is based on the insertion/excision of an insertion sequence element, termed IS*1301*, which is present in multiple copies scattered over the meningococcal chromosome. Although the insertion of IS*1301* is accompanied by a TA duplication, revertants which arise with a frequency of 10^{-4} showed the complete deletion of the insertion sequence along with one copy of the TA duplication, thus reverting the *siaA* reading frame (Hammerschmidt et al., 1996).

A similar mechanism of insertional inactivation of a virulence gene has been found to control *Citrobacter freundii* Vi antigen expression in *E. coli* (Ou et al., 1988). This system however, involves the use of *E. coli* insertion sequence elements and has not been demonstrated to occur in *Citrobacter freundii*.

Unlike the phase variation in the extracellular polysaccharide capsule of the human pathogenic *P. aeruginosa*, its close non-pathogenic marine relative, *P. atlantica*, switches from an extracellular polysaccharide (EPS^+) producing, mucoid phenotype to an EPS^- phenotype. This was shown to occur by the disruption of the *eps* locus by an insertion sequence-like element of 1.2 kb (Bartlett et al., 1988). As is the case in *N. meningitidis*, the insertion sequence completely excises and restores the *eps* reading frame.

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