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COLONISATION AND TRANSLOCATION OF BACTERIA IN THE INTESTINAL TRACT; GENERAL ASPECTS AND STUDIES IN A GNOTOBIOTIC RAT MODEL

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SUMMARY

Bacterial attachment to surfaces or association to other bacterial species is an important subject for understanding the complex bacterial communities that populate the intestinal tract. Disruption of these ecologically stable communities can lead to harmful effects for the host, i.e. permitting the access of opportunistic or newly arrived pathogens to sterile areas of the body producing disease. Virulence factors present in commensal bacteria can be induced in stress situations and could favour translocation. Different colonisation experiments performed in gnotobiotic rats orally administered with pairs of *Escherichia coli* isogenic strains differing in a selected virulence factor suggest a role of P fimbriae and K5 capsule in intestinal colonisation. These *E. coli* traits however, did not favour translocation to mesenteric lymph nodes or other extra-intestinal organs only indirectly by increasing their bacterial numbers in the gut. Gnotobiologic studies are an excellent tool allowing a systemic approach for the study of bacterial traits in colonisation and translocation and should be encouraged for such purposes.

INTRODUCTION

Bacteria grow or attach to almost any surface. Within minutes of exposure of a solid object into sea or freshwater, the object becomes colonised by adherent organisms and the earliest to attach are bacteria (*Beachey*, 1981). Although marine microbiologists have known for long time that bacteria must stick to avoid being swept away, is not long that it has been recognised that adherence is an important ecological trait in the colonisation of specific sites in plants and animals (*Beachey*, 1981; *Zobell*, 1943). Adherence has been shown to be an important requisite in many bacterial infections (*Gibbons*, 1977; *Jones* et al., 1972; *Salyers* et al., 1994). In contrast, the role of adhesion in normal colonisation of the gut has not been proven, thus associations have been observed (*Edmiston* Jr. et al., 1982). An early study by *Hartley* et al. (1979) suggests that the predominant *E. coli* attach firmly to the mucosa. Thus, no mechanism of association was investigated.

BACTERIAL COLONISATION

Important definitions

Bacteria may associate with an inert surface, mucosal epithelium or mucus gel to be able to resist physical removal by washing or peristalsis without a known or specified interaction. Adhesion implies an interaction between a specified bacterial receptor (adhesin) and a corresponding ligand. Colonisation describes a bacterial population that is stable in size over time, implying that the bacteria multiply at least at the same rate as its physical elimination. Normal microbial flora, or more correctly microbiota, consists of a complex microbial community who colonise body surfaces such as the skin, upper airways, oral cavity and intestinal tract. The composition of this microbial population varies depending on nutrient and oxygen availability (*Tancrède*, 1992), where each species inhabits its specialised niche, which manifests as a selectivity in localisation and preferred substrates for metabolism (*Savage* et al., 1968).

BACTERIAL POPULATION IN THE GASTROINTESTINAL TRACT

The gastro-intestinal tract represents the major reservoir of bacteria in the human body. It is estimated that 400-500 different species reaching a total population level of 10^{14} inhabit the large intestine (*Luckey* et al., 1972; *Moore* et al., 1974). The bacterial populations and numbers vary along the gastro-intestinal tract, where the lowest levels are found in the stomach due to gastric acidity, and the highest concentrations are in the large intestine, where the contents are more static. It is important to mention the terms **transient** and **resident** bacteria, the former refer to bacteria that are isolated once, but who do not permanently colonise the intestine. Their presence is temporary resulting from food products or the environment. Residents, on the other hand, are bacteria that normally colonise or persist for long periods of time (*Savage*, 1999; *Sears* et al., 1949).

Ecological niches of the intestine

There are at least 5 microhabitats that can be inhabited by the bacterial population of the intestinal tract:

1. *The surface of epithelial cells.* They are identified by specific binding, often mediated by special organelles, such as fimbriae and afimbrial adhesins.
2. *Deep layer of the mucus gel of the crypts.* Micro-organisms colonising these sites are generally motile and spiral-shaped (*Borrelia*, *Treponema* and *Spirillum* spp.) (*Lee*, 1985). Active chemotactic directed motility towards the bottom of the crypts allows them to traverse the mucus gel and probably resist removal by the mucus flow (*Freter*, 1992).
3. *Mucus gel covering the epithelium.* Mucus is a viscous gel lubricating and protecting the epithelium. It is a mixture of mucin, water, electrolytes, sloughed epithelial cells, digested food components, exuded plasma and proteins. Some bacterial species have the capacity to degrade mucin molecules, notably, *Bacteroides*, *Bifidobacterium* and *Eubacterium* (*Salyers*, 1995). Released oligo- and monosaccharides may provide nutrients for other members of the microbial flora. Studies have shown that mucus is a good substrate for colonising bacteria and that bacteria associate with the mucus (*Cohen* et al., 1983, 1985; *Costerton* et al., 1983; *Guiot*, 1982).

4. *Intestinal lumen*. In the small intestine, where peristalsis is vigorous, bacteria may not persist in the intestinal lumen. In contrast, the colonic lumen contains large numbers of bacteria. It is however, not clear whether the lumenal bacteria are multiplying, or if they represent daughter cells of the actively replicating mucosa associated populations (*Freter*, 1992), since luminal contents are poor substrates for bacterial growth (*Wadolkowski* et al., 1988). In addition, it has been shown that adhesion of an enterotoxigenic *E. coli* strain to tissue culture cells gave a growth advantage compared to a non-adherent strain due to leakage of nutrients from the epithelial cells (*Zafiri* et al., 1987).

5. *Bacterial biofilms*. Indigenous bacteria form a thick multi layered population especially in areas rich with nutrients (*Freter*, 1981). Adhesion to existing micro-organisms rather than to any epithelial surface may be important in such instances. An example of this can be represented by members of the genus *Actinomyces* that adhere to streptococcal species in the oral cavity. Streptococci in turn, binds to the tooth surface (*Cisar* et al., 1979).

Successful association of bacteria to mucosal sites involves a large number of steps. The process, which has been studied mainly in pathogenic bacteria, may require or at least is facilitated by the presence of **virulence factors.** Virulence factors may simply have evolved to permit persistence in mucosal tissues and virulence may be coincidental. The major steps include:

1. Chemotactive attraction of bacteria to the surface of the mucosal gel, which can be facilitated by the production of motile organelles, such as flagella.
2. Penetration of the mucosal gel, as discussed above, may occur passively but can be enhanced by motility and chemotactive gradients.
3. Adhesion to receptors in mucus or the mucosa-associated layers of indigenous bacteria.
4. Adherence to epithelial surfaces, and finally
5. Multiplication of the mucosa associated bacteria (*Freter*, 1981).

The importance of adherence to mucosal cells in pathogenic bacteria may be summarised as follows:

1. Bacterial attachment protects the bacteria from being swept away (i.e. urinary flow in the urinary tract or by peristalsis in the small intestine).
2. Penetration can proceed after adherence to the tissues.
3. Toxic products can be secreted after cell contact or adhesion of the bacteria

We must not forget however, that pathogenic bacteria must overcome a number of local defences before they are able to attach to the epithelial cells.

COLONISATION RESISTANCE

It has been known for long time that normal microbiota limits the persistence of foreign or newcoming bacterial species. Early studies by *Sears* et al. (1951, 1955) show that ingested *E. coli* strains in human volunteers or dogs cannot displace existing resident ones. This restricting capacity of the indigenous microbiota has been known since long and identified by different names during the years, until a team of investigators conclusively established the term colonisation resistance (*van der Waaij* et al., 1971). It is defined as the resistance to colonisation of the alimentary canal by newly ingested micro-organisms (*van der Waaij*: History of recognition and measurement of colonization

resistance of the digestive tract as an introduction to selective gastro-intestinal decontamination. ISGNAS home page: http://www.isgnas.org/isgnas.htm).
The term is now being recognised by clinicians concerned with the negative effect of antimicrobial therapies on the commensal bacteria (*Donnelly*, 1993). The mechanisms controlling colonisation resistance are not completely understood but probably include: competition for substrate, competition for attachment sites, production of bacteriocins which directly kill or inhibit other bacteria, and production of short chain fatty acids. In addition, indirect effects could include stimulation of intestinal motility and mucosal immunity of the host (as revised in *Herías*, 1998).

BACTERIAL TRANSLOCATION

It is defined as the process by which bacteria cross the intestinal barrier and reach the bloodstream or other extra-intestinal sites such as liver or kidneys (*Berg*, 1983). The passage of endotoxin has also been discussed by *van Leeuwen* et al. (1994). The study of translocation has become increasingly important because is being considered as an initial step in the pathogenesis of sepsis, meningitis or other serious conditions that could eventually lead to multiple organ failure and death (*van Leeuwen* et al., 1994). Various studies agree in that the gut bacteria are a principal source of postoperative sepsis, bacteraemia and meningitis in debilitated patients and in neonates (*Lambert-Zechovsky* et al., 1992; *O'Boyle* et al., 1998; *Sarff* et al., 1975; *Tancrède* et al., 1985). Pathogens like *Salmonella*, *Shigella* and *Listeria* as well as members of the normal microbiota, including *E. coli*, *Klebsiella*, *Proteus*, enterococci, staphylococci and lactobacilli have been shown to have the ability to translocate, while obligate anaerobes (with some exceptions like *Bacteroides fragilis* and *Clostridium perfringens*) do not usually translocate (*Berg* et al., 1979; *O'Boyle* et al., 1998; *Steffen* et al., 1988; *Tancrède*, 1992).

Translocation is also considered as an important process for immune priming (*van Leeuwen* et al., 1994; *Wells* et al., 1988). It has been shown that bacteria that are able to persist in the Peyer's patches stimulate a better immune response than those non-persistent (*Hohmann* et al., 1979). The site for bacterial translocation is still a debating issue, but many studies agree that Peyer's patches, specifically through the M-cells seems one of the most likely sites (*Jones* et al., 1995; *Owen* et al., 1986; *Pappo* et al., 1989, *Wells* et al., 1988). Invasion through epithelial cells or passage through tight junctions are also possible, but have been documented mainly for pathogenic bacteria (*Perdomo* et al., 1994; *Rüssmann* et al., 1996; *Savage*, 1972).

With the data mentioned above, I do believe that translocation can be divided or occurs in two different circumstances:

1. During the process of antigen recognition, as a physiological condition and consequent priming of the immune system. This can be supported by studies of (*Shroff* et al., 1995).
2. During catabolic stress, starvation, turgor pressure, altered temperature, antibiotics and osmolality changes in the host that induce strong signals in bacteria which must then struggle to cope and adapt to the harsh environmental changes. As stated by *Alverdy* et. al.: "Harming the host is not the microbe's intent; its goal is to prevail. Injury to the

host by a microbe struggling to survive is the inadvertent consequence of a threatening environment" (*Alverdy* et al., 1994).

IMPORTANT VIRULENCE FACTORS IN *E. coli* (Figure 1).

Fimbriae mediate attachment to host structures. The word originates from the latin meaning "threads" or "fibres" and was introduced by Duguid in 1955 (*Duguid* et al., 1955). In general, they consist almost entirely of protein, are around 7 nm wide x 0.5-2 μm long rigid helical polymers and are found on Gram-negative bacilli (e.g. enterobacteria), Gram-negative cocci (*Neisseriae*) and in some Gram-positive bacteria such as *Corynebacterium* spp. (*Johnson*, 1991, *Ørskov* et al., 1983). **Type 1 fimbriae** are present in about 80% of the wild-type *E. coli*, and are also found in many other species of the family *Enterobacteriaceae* (*Klemm* et al., 1994). They bind to mannose-containing carbohydrate moieties of various human tissues. The exact role of this fimbriae has not been elucidated, but it has been suggested to be involved in cystitis (*Johnson*, 1991). **P fimbriae** bind to oligosaccharides containing an internal or terminal Galα1-4Galα moiety. It is the most important virulence factor for *E. coli* causing urinary tract infection (*Johnson*, 1991). **S fimbriae** mediate binding to sugar moieties comprising sialic acid in α2-3 or α2-6 linkage to lactose in glycoproteins. They bind to many host structures including laminin and brain microvascular endothelial cells (*Hacker* et al., 1994). It is a main factor associated with neonatal meningitis, being present in around 30% of the isolates (*Korhonen* et al., 1985).

Phase variation is defined as a reversible on-off switch in the expression of a property. The phenotypic expression of fimbriae is affected by the bacteria's surrounding environment (temperature, osmolarity, solid or liquid growth media, etc).

Capsules coat the bacterial cell interfering with O-antigen detection and protecting from host immune defence mechanisms. They consist of linear polymers or repeating carbohydrate sub-units that can also contain a prominent amino acid or lipid component (*Jann* et al., 1990; *Johnson*, 1991). There are around 70-80 known capsular (K) antigens in *E. coli.* **K1** and **K5**

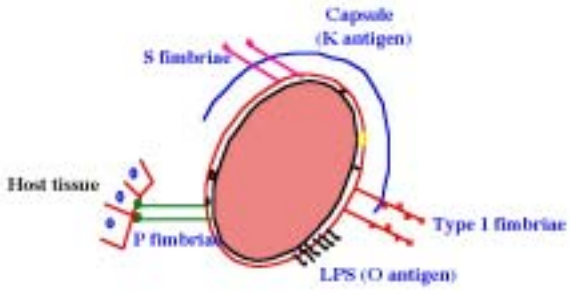

Figure 1: Virulence factors that were studied in *E. coli*. The explanation for each is provided in the text.

Table 1: Identification and characteristics of the bacterial strains used. Each pair includes a different colonisation group and the difference between each one is highlighted in bold letters (Modified from *Herías*, 1998).

E. coli isogenic strains	Serotype	Reference
506 transformants		
506 MS (**Type 1 Fim+**)	O19,22:K1:H-	(*Hagberg* et al., 1983)
506 MR (**Pfim+**)	O19,22:K1:H-	(*Hagberg* et al., 1983)
GR-12 mutants		
742 (**Type 1 fim+**)	O75:K5:H-	(*Svanborg-Edén* et al., 1982)
824 (**Pfim+**)	O75:K5:H-	(*Svanborg-Edén* et al., 1982)
972 (Type 1 +Pfim+)	O-**K5**:H-	(*Svanborg Edén* et al., 1987)
998 (Type 1 +Pfim+)	O-**K-**:H-	(*Svanborg Edén* et al., 1987)
973 (Type 1 +Pfim+)	O75:**K5**:H-	(*Svanborg Edén* et al., 1987)
997 (Type 1 +Pfim+)	O75:**K-**H-	(*Svanborg Edén* et al., 1987)
3034 S-fimbriated mutants		
3034 Sm (**Sfim+**)	O18:K1:H7	(*Pouttu* et al., 1999)
3034-8 (**Sfim-**)	O18:K1:H7	(*Pouttu* et al., 1999)

capsules have been implicated in the majority of extraintestinal infections, because both cross-react with human tissue structures, sialic acid for K1 (*McGuire* et al., 1964) and a precursor of heparin for K5 (*Vann* et al., 1981), which permit them avoid immune recognition.

O antigen is the serologic name given to the lipopolysaccharide (LPS) covering the outer membrane of Gram-negative bacteria. LPS is formed by the O side chains, a core region and lipid A. The antigenic specificity of the O antigen is determined by the composition and linkage of the sugars that form the O side polysaccharide chains (*Hammond* et al., 1984). There are about 164 O antigens typable for *E. coli*, and only a few relative number seem to account for the majority of pathogenic species (*Schiffer* et al., 1976, *Ørskov* et al., 1985).

DO BACTERIAL TRAITS ASSOCIATED WITH VIRULENCE ENHANCE COLONISATION AND/OR TRANSLOCATION IN *E. COLI*? STUDIES IN A GNOTOBIOTIC RAT MODEL.

Pathogenicity can be enhanced by virulence factors, but probably they can also enhance persistence in the intestine as a normal colonisation process or could favour translocation. To study this hypothesis, we colonised germfree rats with *E. coli* strains differing in some recognised virulence factors (*Herías*, et al., 1995; 1997, *Herías* et al., 2001). The approach included **isogenic strains** that are bacterial species that have the same parental origin but differ in the chosen characteristic (*i.e.* capsule or fimbriae). The bacteria were orally administered and allowed to colonise for around 13-15 days.

Importance of type 1 and P fimbriae

Two types of isogenic strains were used to test the ability of type 1 and/or P fimbriae to colonise the intestine of germfree rats (Table 1). The 506 family derived from a human faecal isolate that expressed neither P nor type 1 fimbriae. The strains were transformed with a plasmid conferring either type 1 fimbriae and chloramphenicol resistance (506 MS) or P fimbriae and tetracycline resistance (506 MR) (*Hagberg* et al., 1983). After the colonisation period of two weeks, we observed that the 506 strains were not suitable to test the role of P or type 1 fimbriae for *in vivo* colonisation, because the plasmids enabling the fimbrial expression were lost, and thus, no adhesin advantage could be tested (*Herías* et al., 1995).

The second study included the GR-12 mutants (serotype O75:K5:H-), derived from a pyelonephritic isolate which originally expressed both type 1 and P fimbriae and therefore capable of phase variation. The derivatives used, 742 (expressing type 1 but not P fimbriae) and 824 (expressing P but not type 1 fimbriae), were obtained by chemical mutagenesis (*Svanborg-Edén* et al., 1982). With these strains (see Table 1), both capable of phase variation, it was shown that strain 824 colonised at much higher levels than 742, its type 1-fimbriated counterpart. The difference was highly significant ($p<0.001$), suggesting the advantage of P fimbriae over type 1 fimbriae for persistence in the intestine (*Herías* et al., 1995).

Importance of K5 capsule

The O75:K5:H- family was further manipulated and four mutants were generated differing in the expression of the K5 capsule and the O75 LPS (*Svanborg-Edén* et al., 1987). For the colonisation experiments, we used two different combinations of the strains (see Table 1). In the first colonisation, both strains lacked the O75 antigen, but differed in the expression of the K5 capsule. After colonisation for 11-12 days, the strain expressing K5 (strain 972) reached about 3.8 log higher levels ($p<0.001$) than the K5 negative mutant (strain 998) (*Herías* et al., 1997).

In the second colonisation, the two strains used expressed the O75 antigen, but differed in the K5 expression, where 973 was K5+ and 997 was K5- (See Table 1). After the colonisation period of two weeks, the strain expressing K5 capsule was also established at higher level compared with the K5 negative (1.3 log higher, $p<0.01$). The results were also confirmed by serology (*Herías* et al., 1997).

Importance of S fimbriae

For the study of S fimbriae, we colonised germfree rats of different age groups (adult, infant and neonatal) with two isogenic *E. coli* O18:K1:H7, strain 3034 Sm (which expressed S fimbriae, Sfim+) and 3034-8 (which lacked a functional gene for S fimbriae, Sfim-) (Table 1, (*Pouttu* et al., 1999). *E. coli* reached similar population levels in the colon of all three age groups of rats. The population levels in the small intestine were irregular in all groups, but both neonatal and infant rats had higher levels of *E. coli* than the adult rats (mean 6 vs. 3^{10} log). The Sfim+ and the Sfim- mutants colonised at similar levels in the colon and the small intestine. Thus, no importance of S fimbriae for colonisation was obtained (*Herías* et al., submitted for publication).

Translocation

The dependence of translocation on intestinal bacterial population numbers was also noted in our study, as has been reported before (*Steffen* et al., 1983). Translocation to mesenteric lymph nodes was rarely seen when *E. coli*

colonisation levels were below 1 x 10^8 CFU/g of intestinal contents (see Figure 2). The O75:K5:H- did not usually translocate in high numbers, even if reaching very high colonisation levels. Conversely, the 506 family generally did not reach high levels in the intestinal contents, but translocated in high numbers in the cases when high enough levels were reached in the intestine (*Herías*, 1998).

When comparing isogenic strains, neither K5 capsule, nor P or S fimbriae seemed to influence translocation, other than indirectly by affecting intestinal colonisation levels. In this respect, although all isogenic strains studied had similar translocation capacity, those expressing K5 capsule or P fimbriae by increasing the numbers in the intestine, may indirectly increase translocation (*Herías*, 1998).

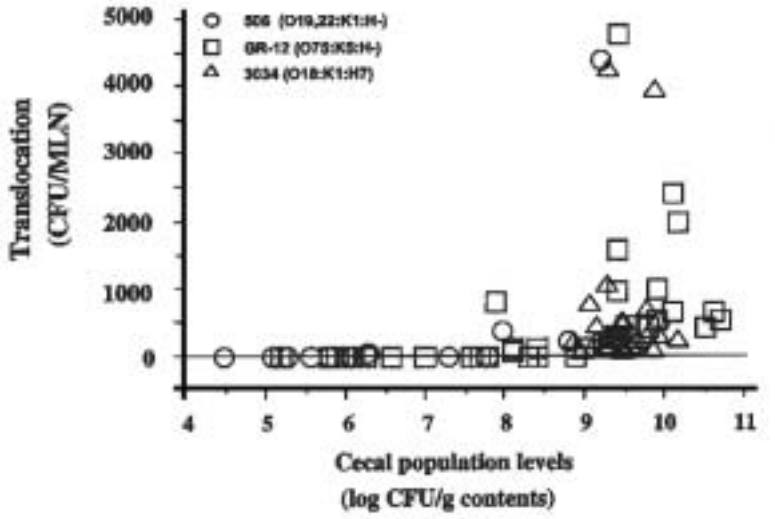

Figure 2: Relationship between bacterial levels in the cecal contents and translocation to mesenteric lymph nodes (MLN). Each symbol represents one rat and one bacterial strain. The different symbols represent the three families of *E. coli* mentioned above. CFU= colony forming units.

CONCLUDING REMARKS

Colonisation of commensal bacteria in the intestine is a complicated process, which includes a serial succession of bacterial species. The bulk of bacteria, which permanently reside in the intestine, include beneficial strains but also potential pathogens. Both bacterial populations live in a balanced ecosystem that when disturbed (by *i.e.* antibiotics or disease) could lead to detrimental consequences to the host.

Bacterial translocation occurs as a normal process for priming the immune system, but it also happens as a consequence of a microbial imbalance. Studies in germfree rats colonised with *E. coli* show that some virulence factors (P fimbriae and K5 capsule) could help for the colonisation process favouring persistence in the intestine. These traits however, did not favour translocation in this model. It will be interesting to determine if factors allowing translocation during a physiological process (if any) are the same allowing potential pathogenic bacteria to invade in stressful or debilitated conditions of the host.

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