Pathogénie moléculaire et cellulaire des infections bactériennes

Leçon 4

Génétique et régulation de la virulence bactérienne : vers la version moléculaire des postulats de Koch

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Molecular and cellular pathogenesis of bacterial infections

Lecture 4

Genetics and regulation of bacterial virulence: towards the molecular version of Koch's postulates

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RESUME : De nombreux gènes qui codent pour des propriétés de virulence des bactéries ont une origine étrangère, comme l'attestent le G+C % et le taux préférentiel d'usage des codons. Après leur entrée dans la cellule bactérienne, ces gènes sont incorporés de manière stable dans le génome bactérien, sur des structures particulières d'ADN qui sont présentes sur le chromosome bactérien ou qui «vivent» dans le cytoplasme bactérien en tant que réplicons indépendants. Dans l'ordre chronologique de leur découverte, les (bactério)phages, plasmides, transposons et îlots de pathogénicité ont été décrits au 20e siècle.

Les phages, ou virus des bactéries, peuvent interagir de deux manières différentes avec la cellule bactérienne : le cycle lytique et le cycle non lytique. Dans le second cas, le phage co-existe de manière stable avec la cellule bactérienne, soit intégré dans le chromosome bactérien, soit comme un plasmide. Ces phages sont appelés phages tempérés et leur forme intracellulaire, prophages. Les plasmides sont des molécules circulaires d'ADN double-brin de petite taille qui «vivent» librement dans le cytoplasme bactérien, bien qu'ils dépendent en partie de la machinerie enzymatique bactérienne pour leur réplication et pour la transcription de leurs gènes. Ils peuvent aussi s'auto-transférer horizontalement entre cellules bactériennes par la conjugaison plasmidique. Les transposons sont des fragments linéaires d'ADN double-brin qui peuvent «sauter», avec ou sans réplication simultanée, du chromosome bactérien sur un plasmide ou d'un site sur un autre site du chromosome. En plus des gènes nécessaires à la réplication, à l'intégration, à la conjugaison et/ou à la transposition, la présence de gènes qui codent pour des adhésines, des toxines, ou d'autres propriétés impliquées dans la pathogénie des infections bactériennes, aussi bien à bactéries Gram positives que Gram négatives, est bien connue depuis de nombreuses années.

La structure génétique la plus récemment décrite est «l'îlot de pathogénicité». Ces îlots de pathogénicité sont des fragments linéaires d'ADN double-brin d'une taille variant entre 10 et 200 kilobases qui sont intégrés dans le chromosome bactérien ou, plus rarement, sur un plasmide de grande taille. Ils sont insérés à hauteur de sites spécifiques d'intégration (ou «hot-spots of integration»), fréquemment situés à hauteur des régions qui codent pour des ARN de transfert. Ils peuvent être perdus par délétion «en bloc». Par définition, les îlots de pathogénicité sont porteurs de gènes qui codent pour des propriétés de virulence prouvées ou suspectées. Ils ont été décrits dans de nombreuses espèces bactériennes Gram négatives, mais aussi dans certaines espèces bactériennes Gram positives.

Quelle que soit leur localisation, l'expression des gènes de virulence est régulée par différents mécanismes, sous l'influence de paramètres extérieurs : molécules chimiques, température, pH, présence/absence

d'oxygène…, ainsi que sous l'influence d'événements stochastiques. Ces régulations se produisent via des réarrangements génétiques, des régulations transcriptionnelles et/ou des modifications post-traductionnelles. Un mécanisme particulier de régulation de l'expression de gènes de virulence est le système de «*quorum-sensing*» dans lequel l'expression des gènes est sous l'influence de la concentration de molécules (auto-inducteurs) synthétisées et sécrétées dans le milieu extérieur par des cellules bactériennes appartenant à la même espèce. Ces systèmes de «*quorum-sensing*» sont le sujet de la 5e leçon.

L'existence de gènes codant pour des propriétés spécifiques qui rendent pathogènes certaines ou toutes les souches d'une espèce bactérienne ont amené Stanley Kalkow en 1984 à proposer la version moléculaire des postulats de Koch, ou postulats de Koch pour gènes : (i) le gène doit être présent dans les souches associées à la pathologie et pas dans les autres ; (ii) la mutation de ce gène doit réduire fortement, sinon abolir, le pouvoir pathogène de ces souches et (iibis) la complémentation en trans doit permettre la restauration du pouvoir pathogène ; (iii) l'expression *in vivo* du gène doit être démontrée ; et (iv) des anticorps spécifiques doivent être produits et conférer une protection, au moins partielle, contre la maladie.

GENETIC DEFINITION OF BACTERIAL PATHOGENS

The bacterial world can be simply subdivided into three groups in relation with pathogenicity : the non-pathogens, the opportunistic pathogens and the primary pathogens. The non-pathogens can live freely in the external environment or in contact with multicellular hosts, on their mucosae and skin. These events have occurred during the millions of years of evolution, since the development of multicellular eukaryotes 500 to 600 million years ago, as mentioned during the inaugural lecture. These associations with hosts do not cause any real damage and are the basis of commensalism or parasitism. There exist facultative commensals, which can live indifferently on the host or in the external environment and obligate commensals, which can live only on the host surfaces.

Amongst commensal bacteria, some have, over the course of time, acquired genes and properties that give them the ability to cause host damage and subsequent disease by accomplishing one or several of the four stages of the development of a bacterial disease, as presented during one of the previous lectures :

(i) colonisation of the epithelia ;

(ii) crossing of the epithelia to penetrate into the mucosa and submucosa ;

(iii) invasion of the host via the blood stream ;

(iv) production of a deleterious effect on the host cells and tissues.

Opportunistic pathogens only cause damage when the host is compromised or injured in some way. Causing host damage is not their natural way of living. Some are also capable of living freely in the external environment outside the host body ; others are obligate commensals that only live in contact with a eukaryotic host.

By contrast, primary pathogens are bacteria that cause damage to the uncompromised host as part of their life cycle. They may be facultative pathogens that are also capable of living freely in the external environment outside the host or obligate pathogens that only reside within the infected hosts and cause disease.

The opportunistic pathogens have acquired some virulence properties during the course of evolution in comparison with the non-pathogens ; the primary pathogens have done the same in comparison with non-pathogens and opportunistic pathogens. On the other hand, the obligate commensals and pathogens have lost genetic material, in comparison with the facultative strains, which was necessary for adaptation to different environments.

LOCALISATION OF VIRULENCE GENES

The purpose of this fourth lecture is to review the origin of such genes, to describe their localisation within the bacterial genome, then to move on to the molecular version of Koch's postulates and finally to explain a few of the regulation mechanisms involved in the expression of virulence genes.

i) Gene circulation

The foreign origin of many genes coding for virulence properties is testified by the G+C% and by the codon preferential usage. Their origin can be other bacterial species, protozoa, yeasts, fungi, or cells of multi-cellular organisms that exchange genetic materials much more often than was believed half a century ago. The primary way of entry of foreign genes is probably transformation, i.e. uptake of nude DNA fragments after passage through the different membranes of the bacterial cells followed by stable incorporation within the bacterial genome. The story of the discovery of transformation is well known to medical students. While studying the virulence of pneumococci in 1928, Griffith discovered that Rough avirulent and acapsular mutants could revert to the original Smooth virulent and capsulated type of strains when in contact with heat-killed Smooth strains. Only in 1944 was the transforming principle identified to deoxyribonucleic acid by Avery, McLeod and McCarthy. After adsorption on the bacterial surface and passage through the bacterial membranes and wall, the DNA must still be stably incorporated within the bacterial genome in order to be transmitted to the next generations.

This incorporation step can occur by legitimate or illegitimate recombination events, depending on the content of the DNA fragment. Legitimate recombination occurs in the presence of sequence homology via crossing-over. Illegitimate recombinations are not based on sequence homology, but on the presence of very specific sequences both on the bacterial chromosome and on the transformed DNA fragment, as in phage integration and in transposition. A fragment of the newly acquired DNA conferring new

properties to this bacterial cell is now stably inherited.

ii) Bacterial DNA

Not all transformed DNA fragments directly incorporate on the bacterial chromosome to transform a kind bacterium into an evil one. Several become part of phages, plasmids, transposons and/or pathogenicity islands, which will be described following the chronological order of their discovery. Let us before remember what the bacterial chromosome is.

Chromosome

The bacterial chromosome is a double stranded circular molecule carrying all the necessary information for the life cycle of a bacterium. The size of bacterial chromosomes varies from roughly 600,000 to 6,000,000 base pairs. In *Escherichia coli*, the size of the chromosome varies between 4.5 and 5.5 megabase pairs. Compared to the laboratory *E. coli* K12 with a size of 4.63 Megabase pairs, the pathogen *E. coli* strains carry up to 20% more genetic information, acquired during different types of DNA transfer. The bacterial chromosome can carry many different genes coding for virulence properties and is also the place of insertion of prophages, transposons and pathogenicity islands.

Phages

Bacterial viruses, or (bacterio)phages can interact with the bacterial cell in two different ways : through the lytic cycle or the non-lytic cycle. In the former case, the virus replicates and kills the bacteria during the excretion process. In the latter case, the phage stably coexists in the bacterial cell, either integrated on the bacterial chromosome or as a free plasmid. Phage integration represents a first form of illegitimate recombination. Such phages are called temperate phages and their integrated form is called the prophage. The prophage can excise and start the lytic cycle under appropriate induction. During the excision process, it can carry along a fragment of the bacterial chromosome that codes, or not, for a phenotype. If the phenotype is any property involved in the pathogenesis of a bacterial infection, the phage can render pathogenic a non-pathogenic bacterial strain during the following integration cycle : this has been called the transduction mechanism of horizontal gene transfer, and was widely used to study the *E. coli* genome during the first years of molecular genetics, now more than half a century ago. Such examples are well known in several bacterial species, such as the diphtheria toxin of *Corynebacterium diphtheriae*, the botulinum toxins C and D of *Clostridium botulinum* and different Shiga toxins of *Escherichia coli*, to cite but a few examples.

Plasmids

Plasmids are double stranded circular DNA molecules of small size compared to the bacterial chromosome, being composed of between a few to several hundreds kilobase pairs. They represent independent free-living forms of DNA present in the bacterial cytoplasm, although they depend on the cell machinery for their replication and gene transcription. They can also transfer themselves between bacterial cells of the same species, those of closely related species, or, for some of them, even those of distantly related species by the so-called conjugation mechanism. This is an important means of horizontal gene dissemination and was the second most widely used genetic tool in the second half of the 20th century. Besides the genes necessary for the control of their replication and partition during cell division and for the transfer by conjugation, the plasmids can carry genes conferring a phenotype to the bacterial cell. The most popular phenotypes are antibiotic and antiseptic resistances, but plasmids can also carry genes coding for many different specific virulence factors : several toxins and adhesins of different pathogenic strains of *Escherichia coli* ; siderophore production and serum resistance of invasive *Escherichia coli* ; invasive properties of *Yersinia*, *Salmonella*, *Shigella*, and enteroinvasive *Escherichia coli* ; botulinum toxin G of *Clostridium botulinum* ; tetanus toxin of *Clostridium tetani* ; capsular antigen of *Bacillus anthracis* and many other examples.

Transposons

Either on a plasmid or on the bacterial chromosome, genes coding for virulence factors can also be located on structures named transposons (Tn). Transposons are linear double stranded DNA fragments which can « jump » from the bacterial chromosome onto a plasmid, or vice versa, or from one site of the chromosome onto another. For this reason, they have been also named « jumping genes ». Transposition is the second illegitimate recombination process. On the transposon, genes conferring a phenotype to the bacterial cell can also be present. Again, the most popular phenotype is antibiotic resistance, but, in some bacterial species, transposons can carry genes coding for virulence factors, though more rarely than do the plasmids. The oligopeptide STa enterotoxin of enterotoxigenic *Escherichia coli*, which was described during the third lecture, is encoded by genes located on the transposon Tn1681. Being located on a transposon enhances the mobility of any gene that cannot only multiply its number of copies, but can also jump from the chromosome to a conjugative plasmid, for example.

Pathogenicity islands

The most recently described DNA structure is the pathogenicity island (Pai). Pais are 10 to 200 kilobase pair long linear DNA fragments integrated on the bacterial chromosome, sometimes on a plasmid. On the chromosome, they are most frequently present in so-called integration hot-spots (or integration sites), located near DNA regions coding for tRNAs and they can be lost « en bloc » by excision. At the same specific site, different Pais can be integrated into different strains of the same species. Integration of a Pai is yet another form of illegitimate DNA recombination process in bacteria. By definition, they carry genes coding for proven or putative virulence factors. They have been described in several bacterial species, such as the *Enterobacteriaceae*. In *Escherichia coli*, five Pais are the best described. The first four of these, Pai1, Pai2, Pai4 and Pai5, carry genes coding for the α haemolysin, P fimbriae and/or CNF1 and are inserted into the *selC*, *leuX*, *pheV* and *pheU* genes, respectively. The fifth of this group of best described Pais, Pai3, carries genes coding for the attaching/effacing lesion, as described during the second lecture, and is inserted into the *selC* or *pheU* genes, depending on the strains. In *Salmonella enterica*, at least three Pais have been described that carry genes coding for cell

invasion, the type III-secretion system, intracellular survival and multiplication and/or enteropathogenicity. Different Pais have also been described in species of *Shigella*, *Yersinia*, *Pseudomonas*, *Listeria*, *Staphylococcus*, *Clostridium*… Being located on a pathogenicity island enhances the stability of any gene in a bacterial cell.

iii) Examples from Escherichia coli

The bacterial species *Escherichia coli* is subdivided into several different classes of pathogenic strains on the basis of the production of particular clinical signs or lesions, or of properties directly or indirectly linked to their pathogenesis. The genetics of the virulence properties of the so-called enterotoxigenic and enterohaemorrhagic strains, or ETEC and EHEC, respectively, will now be described, as examples.

Virulence properties of ETEC

The enterotoxigenic *E. coli* produce fimbrial adhesins and toxins active on the intestine, causing fluid accumulation in the gut lumen and diarrhoea as the main clinical signs. There are three types of toxin : the heat-labile enterotoxin (LT) (a dimeric A-B toxin resembling the cholera toxin with five B subunits activating the adenylate cyclase), the heat-stable a enterotoxin (STa) (an oligopeptide toxin activating the guanylate cyclase) and the heat-stable b enterotoxin (STb) (another oligopeptide toxin of still uncharacterised mechanism of action). Classically, six different types of fimbrial adhesins are described : F2 and F3 produced by human ETEC, F4 and F6 produced by porcine ETEC and F5 and F41 produced by bovine, ovine and porcine ETEC. The genes coding for the three enterotoxins are located on plasmids. Moreover, the STa enterotoxin is encoded by genes located on the Tn1681 transposon, as just mentioned. The genes coding for five of the fimbrial adhesins (F2, F3, F4, F5, F6) are located on a plasmid, while the genes coding for the F41 adhesin are also located on the bacterial chromosome.

The combinations of virulence factors, or pathotypes, of bovine and porcine ETEC and of their respective plasmids were identified by DNA-DNA hybridisation on colonies and on isolated plasmid DNA after separation by agarose gel electrophoresis. The most frequent pathotypes of bovine ETEC are STa/F5/F41 and STa/F5, with a few isolates belonging to other pathotypes (table 1). In all bovine ETEC, the STa- and F5-encoding genes are not only located on plasmids, but are also located on a unique plasmid in each strain (table 1). Conversely, the F41-encoding genes have never been detected on any plasmid. ETEC have also been described in dogs (though less frequently), but not in cats. The most frequent pathotype is : STa/STb, with some isolates being STa or STb, only. No classical fimbrial has been detected so far (table 2). The pathotypes of the virulence plasmids correspond to the pathotypes of the isolates as expected, with the STa- and STb-encoding genes located on a unique plasmid in each corresponding isolate (Table 2).

In porcine ETEC, more pathotypes have been described (table 3). As in bovine ETEC, the STa- and F5-encoding genes are always located on a unique plasmid in each corresponding isolate, while the F41-encoding genes have never been found on any plasmid DNA. The following plasmid pathotypes have also been observed amongst porcine ETEC : STa/F6 in STa/F6, STa/STb/F6 and STa/STb/LT/F4/F6 ETEC ; STa/STb in STa/STb/F6 and STa/STb ETEC ; STb/LT in STb/LT, STb/LT/F4 and STa/STb/LT/F4/F6 ETEC (table 3). In corresponding isolates, the F4-encoding genes are also plasmid-located, but have not been found in association with any other gene. The results demonstrate the existence of two virulence plasmids in STa/STb/F6 ETEC and in STb/LT/F4 ETEC and of three plasmids in STa/STb/LT/F4/F6 ETEC. Moreover, the STa/STb/F6 porcine ETEC are peculiar in being the only ones harbouring two copies on two different plasmids of the STa-encoding gene.

Table 1. Pathotypes of bovine enterotoxigenic *Escherichia coli* (ETEC) isolates and of their virulence plasmids (Mainil *et al*, 1990, 1992)

***E. coli* isolate pathotypes**	**Virulence plasmid pathotypes**
STa+F5+	STa+F5+
STa+F5+F41+	STa+F5+
STa+F41+	STa+
STa+	STa+
F5+F41+	F5+

Virulence properties of EHEC

The class of enterohaemorrhagic *E. coli*, or EHEC, is well known today as a main cause of haemorrhagic colitis and of haemolytic uraemic syndrome in humans. Apart from humans, EHEC strains are also associated with ruminants : they are carried by healthy young and adult ruminants and cause diarrhoea in young calves. The classical pathotype of EHEC strains includes the production of three virulence-associated factors : the Shiga toxins shared with the verotoxigenic strains, the attaching/effacing lesion shared with the enteropathogenic strains and the O157 enterohaemolysin, a pore-forming toxin of the RTX family.

The Stx of *E. coli* are related to the Stx of *Shigella dysenteriae*. As presented during the previous lecture, all Stx are dimeric A-B toxins composed of five B subunits and one A subunit, inactivating protein synthesis by N-glycosidation of the 28S rRNA and causing cell death. Two families, Stx1 and Stx2, and several Stx2 variants have been described. The *stx1*, *stx2*, *stx2c* and *stx2d* genes are present on chromosome-located lambdoid phages, whereas the *stx2e*, *stx2f* and the *stx* genes in *Shigella dysenteriae* are not.

What is often forgotten today is that the phage localisation of *stx1* genes was already observed in the early 1970s, when the Stx were still unknown as such. During conjugation transfer experiments with human enterotoxigenic *E. coli*, William Smith and Margaret Linggood observed the transfer of a heat-labile enterotoxic factor, from the H19 donor strain to a K12 laboratory strain, but could not

Table 2. Pathotypes of canine enterotoxigenic *Escherichia coli* (ETEC) isolates and of their virulence plasmids (Mainil *et al*, 1998a, 1992)

***E. coli* isolate pathotypes**	**Virulence plasmid pathotypes**
STa+STb+	STa+STb+
STa+	STa+
STb+	STb+

Table 3. Pathotypes of porcine enterotoxigenic *Escherichia coli* (ETEC) isolates and of their virulence plasmids (Mainil *et al*, 1995, 1998b)

***E. coli* isolate pathotypes**	**Virulence plasmid pathotypes**
STa+F5+	STa+F5+
STa+F5+F41+	STa+F5+
STa+F6+	STa+F6+
STa+STb+F6+	STa+STb+ STa+F6+
STa+STb+	STa+STb+
STb+LT+	STb+LT+
STb+LT+F4+	STb+LT+ F4+
STa+STb+LT+F4+F6+	STa+F6+ STb+LT+ F4+
STb+	STb+

isolate any plasmid from the recipient strains. Years later, the enterotoxic factor of this H19 strain was identified, at first by Konowalchuk and collaborators, and later by O'Brien and collaborators, not to the classical plasmid-encoded heat-labile enterotoxin LT, but to the Shiga toxin type 1 (Stx1), which is phage-encoded. The transfer observed by Smith and Linggood had occurred by transduction and not by conjugation.

All information necessary for the production of the attaching/effacing lesion and for the intimate attachment is contained on a pathogenicity island inserted into *selC* or *pheU*, as already mentioned during the second lecture and earlier in the present one. This pathogenicity island has been named "Locus of enterocyte effacement" or LEE, or also Pai3. It was described first of all in human enteropathogenic *Escherichia coli* (EPEC), then in human enterohaemorrhagic *Escherichia coli* (EHEC). Using different DNA hybridisation and PCR assays, Frédéric Goffaux and Bernard China (1997, 1999, 2001) studied the LEE of 12 bovine EPEC and EHEC strains. Their conclusions were that (i) the LEE is present in bovine EPEC and EHEC, but in 9 bovine strains out of 12 its insertion site on the chromosome is not *selC* ; (ii) the internal organisation of the LEE in bovine strains is identical to the LEE of human EPEC and EHEC with the *sep* and *esc* genes coding for the type III secretion system located on the left hand side, the *esp* genes coding for the type-III-secreted proteins located on the right hand side and different genes coding for various effectors, including the intimin adhesin and its translocated receptor Tir, located in the middle of the LEE ; (iii) the identity of the *eae*, *tir* and *esp* genes present on the LEE varies amongst bovine EHEC, as amongst human EHEC, and is related more to the serotype, than to the host of origin of the isolate.

A third virulence-associated property of most EHEC is the production of enterohaemolysins. Enterohaemolysins are active on washed red blood cells and inactive on classical blood agar, in contrast with α haemolysin. Different enterohaemolysins have actually been described. The most interesting one is the so-called O157-enterohaemolysin or Ehx which is produced by all EHEC belonging to serotype O157:H7, and by different percentages of EHEC strains belonging to other serotypes. The genes coding for Ehx are located on a ca. 60 Mdal plasmid named pEHEC plasmid. Ehx is a member of the RTX family of pore-forming toxins, just like the α haemolysin, to which it is closely related. Although Ehx is frequent in EHEC and represents an important epidemiological tool, its role in the development of the disease is still purely speculative.

In summary, all genes coding for Shiga toxins are located on the bacterial chromosome, most of them being actually located on lambdoid phages, the genes coding for the attaching/effacing lesion and the intimate attachment are grouped together on the chromosomal LEE pathogenicity island and the genes coding for the O157 enterohaemolysin are located on a plasmid. So far, only transposons have not been involved in the carriage of genes associated with the pathogenesis of EHEC infections.

THE MOLECULAR VERSION OF KOCH'S POSTULATES

i) Definition

The recognition of the existence of genes coding for specific properties rendering some strains of one bacterial species pathogenic, while the other strains remain non pathogenic, led to the formulation by Stanley Falkow in 1984 of the molecular version of Koch's postulates (or Koch's postulates for genes), to confirm that the property encoded by the gene plays a role in the virulence of the bacterial strain and in the pathogenesis of the bacterial infection :

i) the gene must be present in strains associated with the disease and not in others ;

ii) the mutation of the gene must reduce or abolish bacterial virulence and complementation *in trans* must restore it ;

iii) *in vivo* expression of the gene must be demonstrated ;

iv) specific antibodies must be produced and must confer at least partial protection.

ii) The F17 adhesins of APEC in chickens

The avian pathogenic *E. coli*, or APEC, are capable of causing septicaemia and internal organ infections

in young poultry. Their virulence factors are far from being all identified, but P fimbriae are an important colonisation factor of the aerial sacs where the pathogenesis of APEC infections begins. Nevertheless, only 25% of APEC are positive for P fimbriae. Philippe Stordeur (2003) identified other putative colonisation factors, such as F17 fibrillae, Afa-VIII afimbrial adhesin and S fimbriae in a very large collection of 1600 isolates. Although only about 5% of the isolates were positive, we decided to investigate further the pathogenicity of the F17+ APEC and the role of the F17 fibrillae in their pathogenesis. (Stordeur *et al.*, 2004)

To do so meant trying to answer different classical and molecular Koch's postulates. The first classical postulate is often difficult to answer one hundred per cent, but is satisfactorily met in the case of APEC infections and the second postulate is answered providing that necropsy is performed rapidly after death. The next step was therefore to reproduce clinical disease in poultry with F17+ APEC strains. The three P- F17+ APEC strains chosen by Philippe (BEN148, BEN184 and BEN427) could indeed reproduce the clinical signs, the lesions and the bacteriology of the positive control APEC strain producing P fimbriae, in two different *in vivo* infection models in chickens, and were re-isolated in pure cultures. With this first set of experiments, the third and fourth classical postulates were satisfied.

The following step was to answer the molecular postulates with regard to the F17 fibrillae. The answer to the first molecular postulate is similar to the answer to the first classical postulate. To satisfy the second postulate, Philippe (unpublished results) produced mutant and complemented strains in the F17 fibrillal major subunit-encoding gene by the gene disruption technique. One mutant strain was tested *in vivo* in thoracic aerial sac-inoculated SPF chickens and showed very much reduced, if not totally absent, virulence properties. Moreover the complemented strain had almost fully restored virulence properties. The results were obtained by comparing the score lesion, the bacteraemia at 24h PI and the bacterial colonisation of internal organs of the wild-type, mutant and complemented strains, respectively. In addition, work is still in progress to provide answers to the third and fourth molecular postulates. This series of experiments shows that it is still not only possible, but also necessary, to work following to Koch's classical and molecular postulates, before asserting that one bacterium is a pathogen and that one bacterial property is a virulence factor.

EXAMPLES OF REGULATION MECHANISMS

Koch's molecular postulates are often more difficult to meet than their classical counterparts. Two of the most important drawbacks are the absence of stability of genes during complementation studies and the regulation mechanisms of the expression of several virulence genes. The expression of virulence genes can be regulated by random stochastic events causing so-called phase variation, or by tight and complex mechanisms under the influence of external physico-chemical parameters, such as specific chemical concentrations, temperature, pH, O_2 tension, or by the recently described quorum-sensing mechanism that is the subject of the final lecture. The mechanisms of regulation are themselves various : genetic rearrangements by inversion of a DNA fragment carrying the promoter region, transcriptional regulation by methylation of specific sites of the promoter region, post-transcriptional modifications of the messenger RNA by endoribonucleolytic cleavage and/or post-translation modifications of a protein. Dozens of examples are of course possible, but the following ones will be detailed : phase variation of fimbrial adhesins of *Escherichia coli*, iron regulation of toxin production by *Escherichia coli* and *Pseudomonas aeruginosa* and the general regulation of the virulence genes in *Bordetella* species.

i) Expression of adhesins by *Escherichia coli*

The best examples of stochastic regulation mechanisms are again the gene clusters coding for the P and F1 fimbriae of *E. coli*.

P fimbria phase variation

The *pap* gene clusters coding for the P fimbriae comprise 11 genes. The *papA* gene codes for the major subunit, which is anchored on the usher protein encoded by the *papC* gene, with the help of the minor subunit encoded by the *papH* gene. The tip fibrillum is composed of the different minor subunits encoded by the *papK*, *papE*, *papF* and *papG* genes. The *papD* gene codes for the chaperone protein, whose role is primordial in the construction of the fimbriae, as explained during the second lecture. The differences in the numbers of copies of the subunits synthesised per fimbria (hundreds of the major subunit versus one or a few of the minor subunits) originate from the existence of ribosome binding sites, with different affinities upstream of each gene. But the products of two genes, *papI* and *papB*, regulate the general expression of the *pap* operon. *papI* is transcribed in the opposite direction from the *pap* operon whereas *papB* is transcribed along with the other *pap* genes. PapI regulates transcription of the *papB* gene while PapB regulates actual synthesis of the PapA major subunit under the influence of external parameters, such as temperature and carbon source. Moreover, the transcription of the *papI* gene is also influenced by different external parameters and controlled by PapB, resulting in quite a complex regulation network.

But the expression of the *pap* operon is also regulated by a stochastic event. The transcription of the *pap* operon is in fact influenced by the binding of the Lrp global regulator, or « Leucine-responsive regulatory protein », to its target sequence, 5'-GATC-3'. Though its physiological role is unclear, Lrp is involved in the transcriptional regulation of several gene clusters coding for different fimbrial and afimbrial adhesins. The regulation of *pap* gene transcription by Lrp is based upon the methylation state of the adenine residue in its target sequence, by the Dam enzyme or « DNA adenine methylase », since Lrp binds only to unmethylated target sequences. Although not one hundred per cent proven, the following model can be proposed. The promoter of the *pap* operon has two Lrp target sequences, the proximal and the distal, but only binding of Lrp to the distal sequence activates transcription. Just after DNA replication neither target sequence is methylated and Lrp can arbitrarily bind to either. When binding to the unmethylated distal target sequence, Lrp undergoes a conformational change, allowing

the binding of the PapI. This complex causes bending of the DNA with the help of stabilising histone-like proteins, which results in binding of the RNA polymerase and activation of the transcription of the *papI* gene and of the *pap* operon. This leads to a steady state of expression of the Pap fimbriae. This situation lasts till the next generation. On the other hand, when Lrp binds to the unmethylated proximal target sequence, the distal target sequence is methylated by Dam preventing the binding of Lrp and the transcription of the *pap* operon, either because Lrp can not bind PapI or because the DNA can not bend correctly to allow fixation of the RNA polymerase. Each one out of 100 to 1000 daughter cell shows a different expression pattern from the parental cell : this is called the on/off switch or phase variation.

Type1 fimbria phase variation

The *fim* gene clusters coding for the type1 fimbriae comprise nine genes. The *fimA* gene codes for the major subunit, which is anchored on the usher protein encoded by the *fimD* gene with the help of the minor subunit encoded by the *fimI* gene. The tip fibrillum is composed of the minor subunits encoded by the *fimF*, *fimG* and *fimH* genes. The *fimC* gene codes for a chaperone protein. Finally two genes regulate the expression of the *fim* operon : *fimB* and *fimE*, which are transcribed independently, but in the same direction than the *fim* operon. As with the *pap* operon, the expression of the *fim* operon is regulated by external environmental factors and by a stochastic event or phase variation. This phase variation is the consequence of the action on one invertible DNA fragment of either *fimB* or *fim E* gene products that resemble the lambda integrase family of site-specific recombinases.

Both FimE and FimB in fact promote the inversion of this 314-bp segment of DNA located immediately upstream of the *fimA* gene and carrying the promoter region of the *fim* operon : Fim E promotes the inversion in the « off » orientation ; FimB promotes inversion in both orientations, with a preference for the « on » orientation. The action of FimB or FimE is decided by a stochastic event, but the inversion of the DNA fragment is of course also controlled by a number of accessory histone-like proteins, which maintain the DNA molecule in a favourable or unfavourable configuration, under the influence of parameters from the external environment.

The idea behind phase variation depending on stochastic events is that at random switching of gene expression helps the adaptation of the bacterial population to unpredictable environmental changes, at a low cell cost if they do not occur, but at great population benefit if they do. However, not all gene clusters coding for adhesins are submitted to phase variation. For example, the gene clusters coding for the F4 and F5 fibrillae of enterotoxigenic *Escherichia coli* are not. One can hypothesise that their expression is always to the advantage of the producing *E. coli* strains, or certainly not detrimental, even when the circumstances are not appropriate.

ii) Toxins and iron-dependent regulation

The production of toxins by bacteria can also be tightly regulated at the transcriptional level by different external parameters. One well known parameter is the ferrous ion. Though some details of iron-mediated regulation are yet to be understood, a basic understanding of the molecular mechanisms has been gained for several pathogens. The Fur and Fur-like systems, regulating the expression of the Shiga toxins of *Escherichia coli* and of exotoxin A of *Pseudomonas aeruginosa*, will be described.

The exceedingly low availability of iron (10^{-18} to 10^{-9} Molar) in animal tissues limits bacterial growth but is also an environmental signal indicating to the bacteria that it enters into a host. This signal triggers the co-ordinate expression of bacterial genes that promote their growth and survival and are normally repressed when bacteria grow in iron-rich media. This involves not only genes involved in iron uptake, but also those involved in metabolic pathways and genes coding for different toxins (table 4). Thus, iron regulation of toxin production is part of a global regulatory network coordinating bacterial response to alterations in available iron levels.

Shiga toxins

The A and B subunits of the Shiga toxins are coded by two genes separated by 12 nucleotides and forming one operon. Since there is no evidence for a separate promoter for the B subunit-encoding gene, the 5:1 ratio probably results from the existence of ribosome binding sites (*rbs*) with different affinities upstream of each gene. Otherwise, the *stx* genes coding for the different Shiga toxin variants differ in the regulation of their expression. The phage-located *stx1*, *stx2*, *stx2c* and *stx2d* genes are co-regulated with the late promoter of the phage lysis genes. This coupling of expression of the toxin and phage lysis genes may facilitate release of toxin from the bacterial cells. In addition, the expression of *stx1* genes, but not of the *stx2* genes, is regulated by iron concentration, via the iron-regulatory protein, named Fur. The Fur protein of *E. coli* is a member of a large family of iron-dependent regulators found in many Gram negative and some Gram positive bacteria. The Fur protein binds ferrous iron and the iron-bound form binds DNA at the height of a specific consensus sequence, the so-called Fur box (**GATAATGATAATCATTTTC**). Most commonly, Fur binding represses expression of genes, but positive regulation effects have also been reported.

After binding to iron, the Fur protein undergoes a conformational

Table 4. Examples of iron-regulated bacterial toxins

Toxin names	Bacterial species
Diphtheria toxin	*Corynebactetrium diphtheriae*
Shiga toxins	*Shigella dysenteriae* *Escherichia coli*
α haemolysin	*Escherichia coli*
Exotoxin A	*Pseudomonas aeruginosa*
Tetanus toxin	*Clostridium tetani*
VacA cytotoxin	*Helicobacter pylori*
CHO cell elongating factor	*Plesiomonas shigelloides*

change, with formation of a stable homodimeric form. Binding to DNA promoter regions is now possible to repress transcription of the downstream genes. Fur boxes have been identified in the promoters of the *stx1* genes of *Escherichia coli*. In contrast, the promoters of the different *stx2* genes have no Fur boxes and their synthesis is therefore not influenced by iron.

Exotoxin A

The production of exotoxin A, a monomeric toxin with A-B domains, of *Pseudomonas aeruginosa*, like many *Pseudomonas* species virulence factors, is also regulated by multiple environmental signals. In addition to iron, oxygen tension, temperature and the concentration of certain nucleotides influence the amount of toxin synthesised. The iron-dependent regulation system of *Pseudomonas aeruginosa* is a Fur-like system with a Fur-like protein showing homology to the *E. coli* Fur protein. But the promoter of the *toxA* gene itself has no Fur box. The regulation of the *toxA* gene transcription by iron actually represents a more complex cascade than the regulation of the transcription of *stx1* genes in *Escherichia coli*.

The transcription of the *toxA* gene is directly activated by the positive regulator RegA, whose gene promoter has no Fur box sequence either. A second positive regulator of exotoxin A synthesis, PtxR, has also been identified. Despite the presence of a Fur box-like sequence in the *ptxR* promoter, binding of Fur to the region has not been detected.

An additional step in this regulation cascade has been identified with the positive regulation of the *regA* and *ptxR* genes by PvdS. PvdS was first identified as a factor required for siderophore synthesis and represents at last one Fur-regulated factor. The promoter of the *pvdS* gene, in fact, harbours a Fur box and binding of iron-bound forms of Fur has been demonstrated. Again the Fur protein of *Pseudomonas aeruginosa* binds iron at high concentration and, after conformational changes and dimerisation, binds to the Fur box of the promoter of the *pvdS* gene, repressing its transcription. The downstream synthesis of RegA and PtxR is therefore not activated, nor is, even more downstream, the synthesis of exotoxin A. Of course, additional external and cross regulations, some of them being quorum-sensing-dependent, have been identified, rendering the whole network extremely complex.

iii) The BvgAS regulon of* Bordetella *sp.

The essential virulence factors of the different *Bordetella* species are various adhesins, including the filamentous haemagglutinins, pertactin and fimbriae, and toxins, including the adenylate cyclase CyaA, a dermonecrotic toxin and the pertussis toxin, produced only by *Bordetella pertussis*. The independent genes coding for those virulence factors of *Bordetella* sp. form a so-called regulon because they are regulated along with other non-virulence-associated genes by a two gene operon that was at first named *vir*, for « VIRulence », but was later renamed *bvg*, for « *Bordetella* Virulence Genes ».

The *bvg* operon codes for two proteins BvgA and BvgS and the following regulation model has been proposed on the basis of different experimental results. BvgS is a transmembrane sensor kinase protein, sensitive to environmental stimuli and possessing intracytoplasmic kinase activity, and BvgA is the response regulator. Following the perception of an external stimulus, the cytoplasmic domain of BvgS catalyses the phosphorylation of BvgA in a two step reaction. The first step is autophosphorylation from ATP on a conserved histidine residue and the second step is the transfer of the phosphate residue onto an aspartate residue of BvgA.

The BvgA response regulator is a transcriptional activator in its phosphorylated form, which binds as a dimer to high-affinity DNA sites situated upstream of the promoter regions. The genes activated by BvgA form the *vag* regulon (Virulence Activated Genes) and comprise the virulence genes. One of those *vag* genes codes for BvgR, which in turn negatively regulates a second set of genes, the so-called *vrg* regulon (Virulence Repressed Genes). This model is valid for both *Bordetella pertussis* and *Bordetella bronchiseptica*. The respective BvgA proteins are identical and the BvgS proteins are almost identical. The extracellular sensor domain of BvgS, in fact, diverges between the two *Bordetella* species, a situation that may reflect their differences in ecology and in pathology. This Bvg two component regulatory system in *Bordetella* sp. resembles very much different two component « quorum-sensing » regulating systems of several Gram positive bacterial species. This will be described during the final lecture.

QUORUM-SENSING AND FINAL LECTURE

Gene regulation can thus occur at random as in phase variation, but also under the influence of external conditions. Both systems of gene regulation have selective advantages. In phase variation, a subset of cells may be sacrificed if the conditions do not change, but it will assure the survival of the population as soon as adverse conditions occur. And population survival is more important than individual survival in biology in the long term. In gene regulation under the influence of external conditions, individual cells can adapt themselves rapidly to changing environmental conditions. And this is of course also very important for population survival, but in the shorter term. Moreover, when changing conditions are encountered by a few cells in one environment, it is also beneficial to the whole population to get the message quickly, before encountering these particular conditions. This could be achieved by sending a message of any kind to other cells. This kind of cross-talk between bacterial cells of a population of one bacterial species was described in the early 1950s, but fully recognised only in the 1990s. This phenomenon forms the so-called « quorum-sensing » system of regulation of gene expression using auto-inducers, i.e. stimulating molecules produced by other cells of the bacterial population. This will be the subject of the next and final lecture of this Francqui chair.

FURTHER READINGS

BANN J.G., DODSON K.W., FRIEDEN C., HULTGREN S.J. Adhesive pili of the chaperone-usher family. In : Donnenberg M.S. (Ed), *Escherichia coli* : virulence mechanisms of a versatile pathogen. Academic Press : London, 2002, 289-306.

BURNS D.L., BARBIERI J.T., IGLEWSKI B.H., RAPPUOLI R. Bacterial protein toxins. ASM Press : Washington, 2003, 348 p.

CHINA B., GOFFAUX F., PIRSON V., MAINIL J. Comparison of *eae*, *tir*, *espA*, and *espB* genes of bovine and human attaching and effacing *Escherichia coli* by multiplex polymerase chain reaction. *FEMS Microbiol. Letters*, 1999, **178**, 177-182.

CORNELIS G. Les transposons et séquences d'insertion. *Bull. Inst. Pasteur*, 1982, **80**, 3-59.

COUTURIER M., BEX F., BERGQUIST P.L., MAAS W.K. Identification and classification of bacterial plasmids. *Microbiol. Rev*., 1988, **52**, 375-395.

DAVIS B.D. Gene transfer in bacteria. In : Davis B.D., Dulbecco R., Eisen H.N., Ginsberg H.S. (Eds), Microbiology. 3rd Edition. Harper International Edition : Philadelphia, 1980, 137-152.

DOZOIS C.M., CURTISS R. III. Pathogenic diversity of *Escherichia coli* and the emergence of *«* exotic *»* islands in the gene stream. *Vet. Res*., 1999, **30**, 157-180.

FALKOW S. Molecular Koch's postulates applied to microbial pathogenicity. *Rev. Infect. Dis.*, 1984, **10**, S274-S276.

FALKOW S. What is a pathogen ? *ASM News*, 1997, **63**, 359-365.

GOFFAUX F., CHINA B., MAINIL J. Organisation and *in vitro* expression of *esp* genes of the LEE (Locus of Enterocyte Effacement) of bovine enteropathogenic and enterohaemorrhagic *Escherichia coli*. *Vet. Microbiol.*, 2001, **83,** 275-286.

GOFFAUX F., MAINIL J., PIRSON V., CHARLIER G., POHL P., JACQUEMIN E., CHINA B. Bovine attaching and effacing *Escherichia coli* possess a pathogenesis island related to the LEE of the human enteropathogenic *E. coli* strain E2348/69. *FEMS Microbiol. Letters*, 1997, **154**, 415-421.

HACKER J., KAPER J.B. Pathogenicity islands and the evolution of microbes. *Annu. Rev. Microbiol*., 2000, **54**, 641-679.

MAINIL J. Shiga/Verocytotoxins and Shiga/Verocytotoxigenic *Escherichia coli* in animals. *Vet. Res.*, 1999, **30**, 235-257.

MAINIL J. Le point des connaissances sur les entérites à *Escherichia coli* des veaux. *Ann. Méd. Vét.*, 2000, **144**, 121-136.

MAINIL J. Les souches pathogènes d'*Escherichia coli* chez les chiens et les chats : IV) discussion générale. *Ann. Méd. Vét.*, 2002, **146**, 219-224.

MAINIL J. Facteurs de virulence et propriétés spécifiques des souches invasives d'*Escherichia coli*. I) les adhésines et facteurs de colonisation. *Ann. Méd. Vét*., 2003, **147**, 105-126.

MAINIL J.G., BEX F., DREZE P., POHL P., KAECKENBEECK A., COUTURIER M. Replicon typing of virulence plasmids in enterotoxigenic *Escherichia coli* isolates from cattle. *Infect. Immun.*, 1992, **60**, 3376-3380.

MAINIL J.G., BEX F., JACQUEMIN E., POHL P.H., COUTURIER M., KAECKENBEECK A. Prevalence of four enterotoxin (STaP, STaH, STb, and LT) and four adhesin subunit (K99, K88, 987P, and F41) genes among *Escherichia coli* isolates from cattle. *Am. J. Vet. Res.*, 1990, **51**, 187-190.

MAINIL J., BEZ S., JACQUEMIN E., KAECKENBEECK A. Les souches pathogènes d'*Escherichia coli* chez les chiens et chats : I) détection des souches entérotoxinogènes (ETEC), entéropathogènes (EPEC), vérotoxinogènes (VTEC), entérohémorragiques (EHEC) et nécrotoxinogènes (NTEC). *Ann. Méd. Vét.*, 1998a, **142**, 39-46.

MAINIL J.G., DAUBE G., JACQUEMIN E., KAECKENBEECK A., POHL P.H. Typage des *Escherichia coli* isolées d'intestins de porcelets en Belgique au moyen de sondes génétiques : souches entérotoxinogènes, vérotoxinogènes ou entéropathogènes. *Ann. Méd. Vét.*, 1995, **139**, 5-13.

MAINIL J. G., DAUBE G., JACQUEMIN E., POHL P., KAECKENBEECK A. Virulence plasmids of enterotoxigenic *Escherichia coli* isolates from piglets. *Vet.Microbiol.*, 1998b, **62**, 291-301.

OFEK I., HASTY D.L., DOYLE R.J. Bacterial adhesion to animal cells and tissues. ASM Press : Washington, 2003, 416 p.

SALYERS A.A., WHITT D.D. Bacterial pathogenesis : a molecular approach. 2nd Edition. ASM Press : Washington, 2002, 539 p.

SAUNDERS J.R. Bacterial plasmids and their association with virulence. In : Duffy G., Garvey P., Coia J., Wasteson Y., Mc Dowell D.A. (Eds), Verocytotoxigenic *Escherichia coli* in Europe. 3. Pathogenicity and Virulence (Proceedings). Concerted Action CT98-3935. National Food Centre : Dublin, 2000, 118-125.

SCHMIDT H., HENSEL M. Pathogenicity islands in bacterial pathogenesis. *Clin. Microbiol. Rev.*, 2004, **17**, 14-56.

SMITH H.W., LINGGOOD M.A. The transmissible nature of enterotoxin production in a human enterotoxigenic strain of *Escherichia coli*. *J. Med. Microbiol.*, 1971, **4**, 301-305.

SNYDER L., CHAMPNESS W. Molecular genetics of bacteria. ASM Press : Washington, 1997, 504 p.

STORDEUR P., BEAUPAIN N., MAINIL J. Caractérisation génotypique de souches pathogènes aviaires d'*Escherichia coli* isolées en Belgique. *Ann. Méd. Vét.*, 2003, **147**, 275-280.

STORDEUR P., BREE A., MAINIL J., MOULIN-SCHOULEUR M. Pathogenicity of *pap*-negative avian *Escherichia coli* isolated from septicaemic lesions. *Microb. Infect.*, 2004, **6**, 637-645.

STORDEUR P., MAINIL J. Colibacilloses aviaires. *Ann. Méd. Vét.*, 2002, **146**, 11-18.

STORDEUR P., MARLIER D., BLANCO J., OSWALD E., BIET F., DHO-MOULIN M., MAINIL J. Examination of *Escherichia coli* from poultry for selected adhesin genes important in disease caused by mammalian pathogenic *E. coli*. *Vet Microbiol.***,** 2002, **84**, 231-241.

TOTH I. Phages and their role in virulence of bacteria. In : Duffy G., Garvey P., Coia J., Wasteson Y., Mc Dowell D.A. (Eds), Verocytotoxigenic *Escherichia coli* in Europe. 3. Pathogenicity and Virulence (Proceedings). Concerted Action CT98-3935. National Food Centre : Dublin, 2000, 1-10.