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Epigenetic and Non-epigenetic Switch Mechanisms in *Escherichia coli*

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Switch mechanisms (Phase variations) exemplify transient hypermutatability that help microbes to survive without risks in stress conditions. These processes give rise to temporary diphasic and multiphasic expressions that are heritable yet capable of fluctuating back with modulating frequency. Surface proteins like Ag43, fimbriae & pili of *Escherichia coli* have shown to phase vary with underlying epigenetic and non-epigenetic strategy. Expression of Ag43 and Pap P are under the control of methylation dependent systems with mutual exclusion and differential binding as key processes. Fimbriae I "flips" between fimbriated and non-fimbriated state by conservative site specific recombination events.

Key words: Switch mechanisms, Epigenetics, Phase variation, Colony morphology, Transient hypermutation.

Bacteria are nature's minuscule fighters that show uncanny ability to survive against inundated stress factors that are often erratic and unprovoked [1,2]. It's a "*Red queen's race*" for most bacterial species[3] to meet these capricious threats, which range from environmental (destabilizing their functionality) to antibiotic stress (challenging their mere survival). The effective counter mechanisms so developed, were acquired or inherent, ubiquitous or species-specific, that enabled the microbe to respond, survive, perpetuate and evolve [2,4]. In absence of any generic backup, stress-induced mutational events not only generate tolerance but also pave way to increased mutation in the selected cells [5,6]. In spite of generating diversity and adaptability, these response measures are disadvantageous due to accumulated mutations that are often harmful[7]. Hypermutability without risks (transient) is a nonpareil choice during this commination [8]. Mutations, that fail to persist or that reverts back, once the threat has elapsed are scenarios that can be categorized under these events. Such temporary reversible responses are "programmed events" which are heritable, with modulated switch frequency in future generations[9]. These situations arise when stochastic events are localized or restricted to certain loci of pan-genome, termed as 'contingency' genes prompting the bacterium to "shift" or "switch" to a favorable form (either at a structural or functional level) for niche adaptation or virulence[10,11].

These switch mechanisms are preliminary evident as colony variants, rooting for the possibility of surface-associated proteins as ideal candidates for phase variation[12]. Cell surface

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appendages like capsules, pili, flagella and functional groups of the cell membrane are reported to exhibit switch mechanisms[13,14]. Clonal population of "phased" cells appears to shift between interconvertible diphasic morphovars of dry/ moist, frizzy/smooth and opaque/translucent or between fimbriated/non-fimbriated and piliated/non-piliated expression. The phase change is brought about by highly tuned regulative events that are either epigenetically or non-epigenetically controlled[16]. These regulative strategies results in either switching the gene/s "off" (none) or "on" (all) thereby preventing the expression of protein/s (phase variation) or that leads into the expression of an alternative surface receptor (antigenic variation). This concept should not be confused with phenotypic "phase variation", an irreversible change brought about by environmental regulation, selection or unidirectional mutation[3]. These events are not only targeted at the surface epitopes alone; but also in restriction modification systems[16] that cause preference or change in sequence specificity of restriction enzymes (phasevarion), regulatory proteins and metabolism associated genes[17].

Molecular mechanism of phase variation

Gene expression is a cumulative play between regulatory proteins and DNA sequences which are intrinsically tuned to work in a step-wise manner to decode four letter blue prints into protein. Sequence alignment, orientation, consensus frequency and spacing between regulatory sites are of utmost importance for a successful expression. Underlying contrivance for phase variation in bacterial species is far from universal with no correlation between phase-varying phenotype and regulatory mechanisms[3,15]. Phase variability resulting due to DNA rearrangements or difference in random repeats (shufflon) is under non-epigenetic control mechanisms. Misalignment of multiple contiguous repeats of short sequence (e.g. microsatellites), between the daughter and parent DNA strands during replication can result in "slippage errors" or slipped strand mispairing (SSM). Microsatellite polymorphism is seen when a repeat is inserted with "backward slippage" (looping back of daughter strand) or with a repeat deducted during "forward slippage" (parental strand loop back). If these repeats are localized at primary regions for transcriptional and translation, any changes in the repeats can disrupt the reading frame that may lead to SSM-dependent phase variability[18, 19].

A unidirectional exchange between allelic forms of a gene sequence by recombinational event can render an expression that is functional or silent. Recombinational events differ by preference for sequence homology and protein dependence. Phase variation based on transpositional events within the structural genes causes disruption of the reading frame and represses expression if it is localized at regulatory regions[20]. General recombination associated phase variation appear to require less homology in the target region and show increased recombinational frequency that differ from the usual rec-dependent recombination[15]. Conservative site specific recombination (CSSR) involving specific recombinases results in inversion of a DNA segment, disrupting the spatial orientation of genes prompting a switch between on (correct orientation) and off (incorrect or reverse orientation) control [21].

Epigenetic modes of phase variation involve modifications that occur without altering the basic sequence of the intended regions[22]. These procedures are dependent on differential preference of the regulatory protein and the ability of the target site to undergo methylation.

Phase moieties in *E.coli*

Escherichia coli, a "*numero uno*" nosocomial UTI pathogen is preliminary "spotted" on Mac Conkey (MaC) or Eosin Methylene Blue (EMB) agar before biochemical categorization. The candidate microbe appears as round pink colonies on MaC whereas they emerge as green metallic sheen colonies in later[23]. Even though individual species exhibit colonies of characteristic size and appearance, there are instances where different morphovars are observed on streaking from pure bacterial clonal culture[24]. With respect to surface properties, *E.coli* adapts two major forms: rough and smooth (based on their O antigen) or frizzy or glossy (based on auto-aggregation and presence of fimbriae). Lipopolysaccharide with a complete core & intact O antigen gives rise to smooth colonies that are concave and circular, but in its absence gives rough colonies that are flat and irregular[25]. Insertion of a transposable element, IS5 within the *rfb* gene cluster controlling O antigen

biosynthesis causes the disruption of reading frame resulting in rough morphology. Reversion of the rough to smooth forms is sporadic in *E.coli*, as this requires the integrity of the *rfb* gene cluster to be restored making it less irreversible [26].

Some other surface proteins are accountable for the reversible, phase-variable colony morphovars in *E.coli* that exhibit diphasic frizzy and glossy morphovars. Immunoflourescent and Phase-contrast microscopy studies traced the frizziness and colony size of the morphovars to Ag43 and fimbriae respectively[27]. Both these surface proteins showed marked differences in regulative mechanisms for phase-variation; with epigenetic regulation by methylation in Ag43 and CSSR system in fimbriae variability. Bacterial strains showing antigenic variation of P, S and CS31A fimbriae have methylation dependent strategies [28].

Non-epigenetic Phase regulation in *E.coli*

Type I Fimbriae Phase variation

Type I fimbriae are widespread among *E. coli* strains as well as in other members of the *Enterobacteriaceae*[29]. These proteinaceous chaperone-usher assembled appendages confer mannose-sensitive adhesion to receptor molecules thus helping in surface recognition and attachment[30]. Studies have demonstrated the association of Type I fimbriae with nearly almost all virulent strains of *E.coli* emphasizing their importance and prevalence in mediating invasion, attachment and colonization and association with pathogenic islands[31]. The expression of this appendage is under operon control of *fim* cluster and phase variation (switching between fimbriated and non-fimbriated state) is mediated by CSSR[32]. The particular operon consists of six structural genes, an invertible *fim* switch region (*fim*S) and regulatory region comprising of recombinases genes (Figure 1-a). The promoter sequence of structural genes lies within the invertible region of 314 base pair (bp), located upstream to *fim*A, the first and main structural subunit of the cluster (Figure 1-b). Proper orientation of promoter region results in fimbriated state or expression ("on" orientation, Figure 1-c) and the "flippage" prevents the transcription of *fim* A leading to a non-fimbriated stage ("off" orientation, Figure 1-d). The inversion element (296bp) is flanked by two inverted repeats (IR of 9 bp each) which are binding sites for recombinases Fim B (IRR) and Fim E (IRL). The inversional event is controlled and accomplished by these site-specific recombinases, which differ in specificity and activity[15]. FimB arbitrates inversion in both directions optimally between 37^0C and 40^0C whereas FimE mediates the inversion predominantly to the "off" orientation at 37^0C. The frequency of inversion mediated by FimE show two to three fold increase in comparison to FimB-mediated inversion, suggesting that relative amount of these two recombinases affect the net phase variation rate of Type I fimbriae[28]. Several physical and cellular factors like Histone like nucloeid structuring protein (H-NS), Integration host factor (IHF), Leucine–responsive regulatory protein (Lrp) are reported in regulating the transcription of these genes. Lrp binds specifically to three sites located within the switch region; formatting a synaptic complex for recombination which further in concert with IHF and H-NS promotes DNA bending. These topological changes allow the IR repeats to be brought into close proximity so that recombinases act to invert the gene orientation [32, 33].

"On" and "off" phase variation in CS18 fimbriae (*fot* operon) found in ETEC (Enterotoxigenic *Escherichia coli)* strains are also accounted by CSSR with products of *fot* S and *fot* T aiding in the inversion of 312bp stretch upstream to *fot* A structural gene. Fot S resembled FimE in aiding both "on" and "off" orientation whereas Fot T resembled FimE showing bias towards the "off" orientation[28].

Epigenetic regulation of Phase variation in *E.coli*

PapP Phase variation

Pap or pyelonephritis-associated pili (P pili) are mostly expressed by uropathogenic isolates of *E.coli* as they exhibit specific binding affinity to digalactoside-containing glycolipids on the uroepithelium[34]. In contrast to the physical inversional event seen in fimbriae, the phase variations in these pili are brought about by methylation[35]. The pap operon comprises of seven structural genes and regulatory region upstream to pBA promoter (Figure 2-a) and depends on the regulatory action of Lrp, PapI, PapB and catabolite activator protein (CAP) for phase variation. A well organized regulatory region inherent with sites (GATC) for methylation enabling preferential binding is also a requisite[36]. The P*ap* regulatory

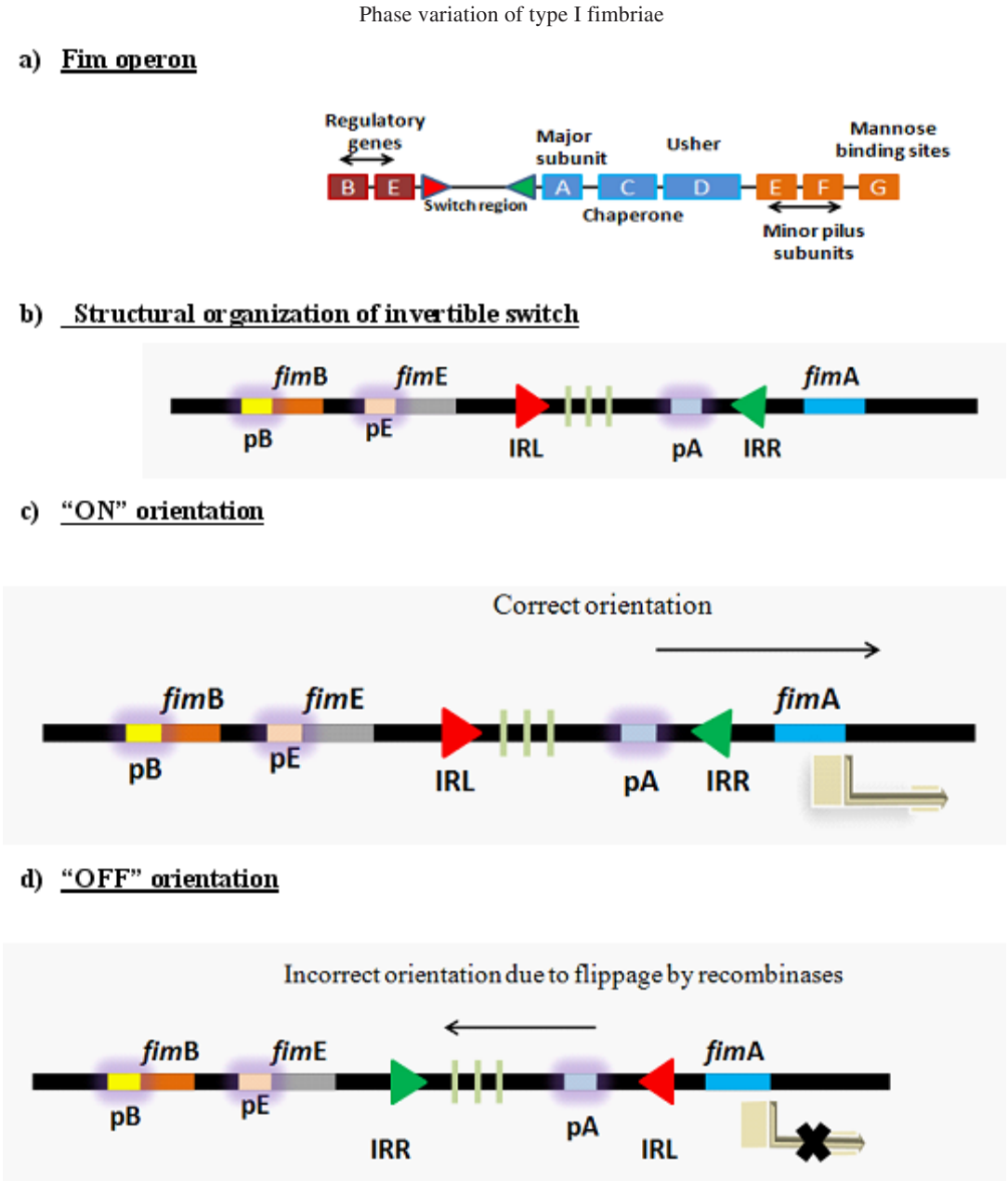

Fig. 1. Schematic representation of Type I fimbriae phase regulation by the inversion mediated by SSM (a) Gene cluster of Fim genes with structural genes, switch region(invertible element) and regulatory region. (b) The relative positions of the promoters (glow boxes), genes (open rectangles), and inverted repeats IRR and IRL (triangles) of *fim* operon, three Lrp binding sites(green lines) are shown (c) "On" phase: correct orientation of the "invertible DNA sequence" allowing the RNA pol to bind with the pA promoter for the expression of FimA.(d) "Off" phase: Flipping or Inversion of the element incorrectly orients the promoter site and prevents the expression of FimA. The drawing is not to scale and is not meant to convey the protein size, biochemical properties or the distance between the target sites.

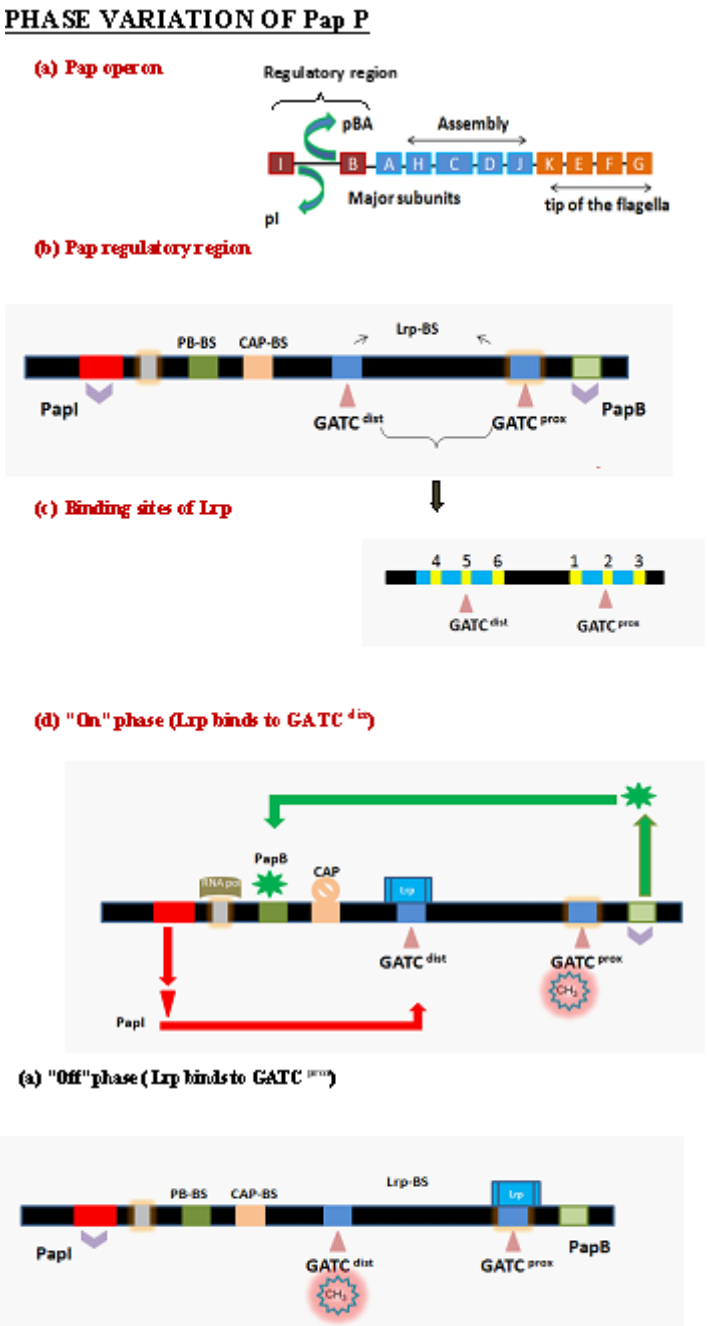

Fig. 2. Schematic representation of Pap phase regulation: (a) pap regulatory region (416bp) with two promoter (pI and pBA), expression of regulatory proteins (Pap1 and PapB) and two target sites (GATC^{prox} and GATC ^{dist}).(b) In the absence and presence of methylation at GATC ^{dist} and GATC ^{prox} respectively, regulatory proteins PapI and CAP helps in binding of Lrp inducing the "on" variation(c) of the six Lrp sites the methylation targets the GATC sequence within the 2nd and 5th site for GATC ^{prox} and GATC ^{dist} respectively. The 2nd site overlaps the consensus sequence for the *papB* gene (d). Methylation at GATC ^{prox} prevents the Lrp binding which causes Lrp to bind with non-methylated GATC ^{dist} turning the phase "on". (e) Methylation at GATC ^{dist} prevents the Lrp binding which now allows Lrp binding to non-methylated GATC ^{prox} preventing the RNA pol binding and initiating the transcription of PapB thus switching "off" the phase. The drawing is not to scale and is not meant to convey the protein size, biochemical properties or the distance between the target site

region (Figure 2-b) of 416 bp comprises of [i] two promoter regions pI (PapI) and pBA (main promoter for *pap* operon) [ii] two targets (GATC) sites for methylation, that are 102bp apart, one distal to the main pBA promoter (GATC ^{dist}/GATC1028/ GATC-I) and the other proximal to the pBA promoter (GATC ^{prox}/GATC1130/GATC-II). The mechanism of molecular switch involves differential binding of a regulatory protein between two methylated target sites[37]. Here, the prime regulatory protein is Lrp, which act as a repressor or activator based on the site of binding. The "on" phase is triggered if the Lrp is bound to the GATC ^{dist} but switches to "off" phase on binding to GATC ^{prox} (Figure 2 - d & e). Differential binding of the Lrp is positively facilitated by PapI and CAP and negatively via DNA methylation of target sites [38]. A potentially critical feature of this system is "mutual exclusion" in which Lrp binding at one region in the regulatory site decreases the affinity for the second site. A total of six sites (Figure 2-c) are present for Lrp binding of which 2nd (proximal to pBA) and 5th (distal to pBA) are the site for methylation, facilitated by Dam (deoxyadenosine methylase). Methylation prevents the attachment of Lrp such that a fully methylated GATC-II are phase "on" cells and "off" phased cells have fully methylated GATC-I region. Apart from the regulatory mechanisms mentioned above, PapB and cAMP-CAP help in regulation, with former activating the pI and latter activating transcription at both the pI and pB promoter regions[15]. Mechanism of phase variation of operons *daa* (F1845 pili), *sfa* (S pili), *clp* (CS31A) are also dependent on the global Lrp and Dam with slight variations in the events [15, 39, 40].

Ag43 Phase variation

Ag43, an outer membrane protein, which belongs to the family of autotransporter causes autoaggregation, enhances biofilm formation, and affects phage adsorption[41]. The expression of Ag43 gives flat, frizzy irregular colonies that autoaggregate in liquid medium, but its absence

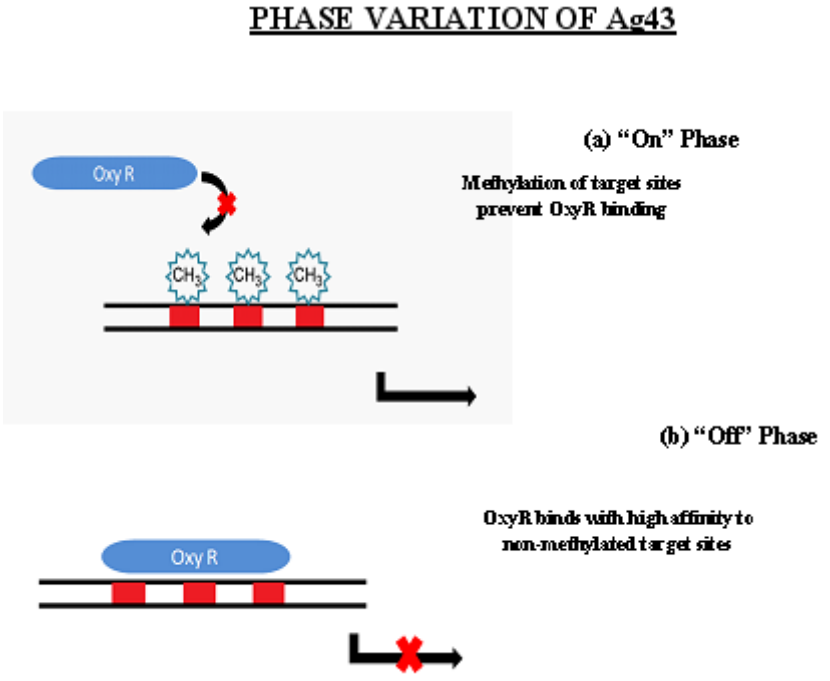

Fig. 3. Schematic representation of Ag43 regulation: (a) The three target sites (given as red blocks) on methylation by Dam prevents the binding on OxyR (repressor) and leads to "on" phase (b) In the absence of methylation in the target sites(GATC),OxyR has great affinity to bind at this site resulting in turning "off" phase. The drawing is not to scale and is not meant to convey the protein size, biochemical properties or the distance between the target sites

causes circular and glossy colonies without any sign of fluffing. Ag43 is decoded from *agn*43 (43 min from the origin of replication) and studies showed that deletions in *oxyR* or *mor* gene (~ 89-min region) "fixes" the cell as frizzy[42]. OxyR is a peroxide sensor and transcription regulator, which can sense the presence of reactive oxygen species and induce antioxidant system in bacteria[43]. The mechanism of phase variation in Ag43 is under epigenetic control but unlike pap it is less complex and only requires Dam and OxyR as key players. Ag43 expression is negatively controlled by OxyR and positively by Dam methylation of agn, the regulatory region[44]. *agn* contains three GATC sequences that are localized within the binding site of OxyR [repressor] and the whole switch mechanism is regulated by the competition between OxyR and Dam for the regulatory region[45]. Ag43 expression is switched "on" (Figure 3-a) when target sites are methylated and "off" (Figure 3-b) when OxyR is bound to the target sites [non-methylated state]. Four forms of interconvertible morphovars are distinguishable by their overall colony appearance, population dynamics and their ability to form cell clumps in liquid media. The forms have resemblance to rough and smooth colony type but have more defined population dynamics showing seeding or homogenous distribution in growth medium. Form 1 is characterized by large, flat, frizzy colonies whereas form 2 is small, convex, glossy colonies. Form 3 resembles form 1 with the former having a smooth surface. Form 4 appears as small convex colonies but with a frizzy surface. Transition between form 1 to a form 2 colony types and vice versa rarely occur directly, but were seen to occur via the form 3 or form 4 colony types suggesting that Form 1 and 2 are predominant forms whereas form 3 and 4 are intermediatory. Immunofluorescence microscopy employing specific sera, it became clear that the four forms coincided with specific Ag43 and Fim phenotypes: form 1, positive for Ag43 and negative for Fim1; form 2 vice versa to form 1; form 3, both negative to Ag43 and Fim1; form 4 positive to both Ag43 and Fim1 [27]. Studies on population dynamics showed that glossy colonies showed opalescent films on dynamic culture and thick surface pellicles in static culture. Frizzy morphovars showed seeding in both dynamic and static culture. Intermediate forms 3 showed homogenous growth in static whereas form 4 resembled form 2.

Studies have shown that fimbriae blocks autoaggregation as the physical presence of fimbriae on the cell seems to prevent intracellular Ag43-Ag43 interaction[46]. The Ag43 or OxyR status does not appear to influence fimbriae expression, indicative that these as exclusive processes [27].

Diagnostics with Phase variation

The potential of phase variation as a diagnostic measure can be implemented for generating rapid phenotypic variability in a clonal population. Epidemiological studies exploring the association of specific clinical symptoms or virulence to phase-variable gene expression in the bacterium can aid in finding markers for understanding pathogenesis. These studies will also give insights into the role of regulatory proteins, their target sites and molecular mechanisms which can help in comprehending the type of survival or adaptive strategy in the bacterium (species) of interest. Majority of identified phase variable moieties are of surface proteins that aid in adhesion, invasion and colonization of the host cells. If a particular phase variant is "fixed" or frequently seen for a pathogenic strain then vaccines can be targeted against these surface proteins for control and prevention.

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