Diplococcic Streptococcus Cleavage, an Ideal Target to Prevent Bacterial Reproduction (Family Planning)

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ABSTRACT

Alterations of penicillin binding proteins (PBP) by a series of point mutations in the pbp genes of Diplococcic Streptococcus chromosome (2300 Kb approx) are apparently responsible for antibiotic resistance crisis. In order to develop a preventive treatment, we should not forget that these patients, children and the elderly are not competent enough for immune response and therefore the available vaccines even polyvalent ones may not always be effective. Most recently, we have recognized that the bacterial reproduction and maturation are regulated by the two –component signaling system via diplococcic streptococcus cleavage. Thinning of cell wall by the growth in Xylitol eliminates cleavage and ideally fulfils our requirement for a low-cost preventive therapy to save human lives and their miseries from bacterial pneumonia, meningitis, otitis media and dental caries.

INTRODUCTION

The diplococcic *Streptococcus pneumoniae* is a serious Gram-positive pathogen which has been used to develop the concept of modern bacterial genetics. Dr Griffith's original work published in 1928 has dealt with difference between the smooth and rough colonies of *Pneumococci* and their difference in pathogenesis[(1)]. It is becoming increasingly clear that Dr Griffith's smooth and rough colonies have nothing to do with the entry of undefined double strand donor DNA fragments as reported by Avery et al, 1944[(2)]. Evidently, DNA macromolecule of Watson and Crick(1953)and the DNA molecule containing genetic information in three base codons as discovered by Khorana, Nirenberg and Holley (1968) confirms that Avery et al have not considered DNA as a bio-macromolecule which means a single chromosome of the Pneumococcus containing all their inheritable genetic characters[(3)]. The TCA insoluble DNA fragments isolated by phenol extraction procedure are usually too small to contain any biologically inheritable character (Palchaudhuri et al, 1972)[(4)]. Based on evidence we accept that the progress in Gram-positive Pneumococcus genetics has been obscured by the in-vitro transfer of double stranded DNA fragments via a plasmid or phage vectors in the laboratory strain of Gram-negative E. coli K-12[(5)]. Many academic Institutions have tilted towards such bacterial gene cloning experiments with Gram-negative E. coli K-12 and drifted our attention far away from the concept of natural transformation of Pneumococcus. For the past 24 years, the mortality rate of children and the elderly from bacterial Pneumonia is increasing at a very high rate even in the presence of our antibiotics, old or new. Taking the entire subject together we like to generalize that StkP plays an important role in S.pneumoniae biological continuity and therefore PASTA domain is the primary target to find a remedy against bacterial lobar pneumonia[(6)]. How much do we know about biological multiplication of S.pneumoniae and its closely related diplococcic bacteria? Not enough to answer all the questions but the diplococcic Streptococci grow in three phases, pre-competent, competent and post- competent[(7)].

In the recent years, it has been accepted that the competent – phase is regulated by the two – component signal transduction system of the live Streptococcus recipient[(8-11)]. We have confirmed such a difference between the competent Pneumococcus and their incompetent derivatives but a fraction of the incompetent population is irreversibly old. Our optical microscopy of mitis group S.oralis after Gram-staining clearly demonstrates that the stationary phase or latent phase contains their total population in a long chain with size heterogeneity, 1.7um to 0.2um as shown in Fig. 1.

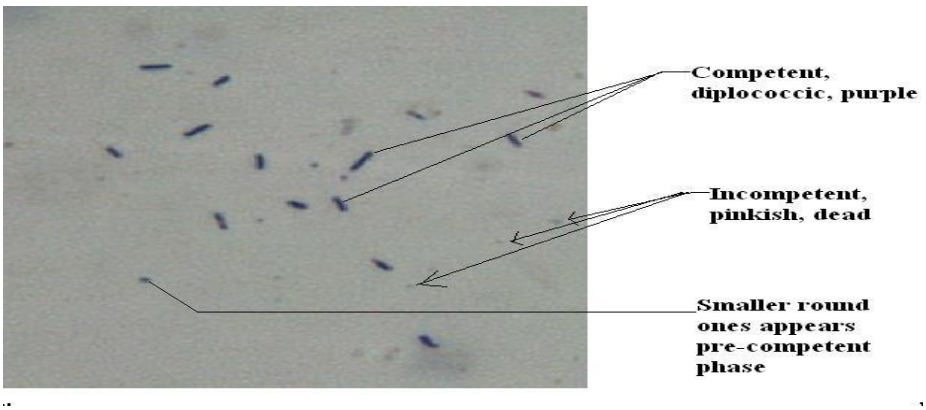

Fig. 1: Stationary phase viridans group *Streptococci,* diluted 10,000-fold in rich broth, allowed to grow at 37°C and then visualized by optical microscopy after Gram staining (magnification 1000X). Bacterial population maintains heterogeneity of sizes; both competent and incompetent bacteria are present. They show both purple and pink with heterogeneity of their sizes

We have accepted the difference between the competent and the incompetent phases of this bacterium but ignored the pre-competent phase. The pre-competent bacterium with its spherical shape (small in size) gradually grows in size and changes to an oval shape. In the pre-competent phase, diplococcic bacterium is born as a small sphere and gradually grows to an oval shape with a thick cell wall. Then bacterium enters into the competent phase with the formation of a cleavage at the mid-position[12]. Prior to this competent phase the bacteria cannot form colony but their growth in size still in pre-competent phase can be easily measured by suspending them in broth or buffer and measuring the optical density (Palchaudhuri S., unpublished data). Growth in size apparently is their cell wall synthesis and unlike E.coli K-12, the Streptococcus cell wall is not a single a layer but a thick cell wall which is gradually synthesized with the increasing number of peptidoglycan layers and cross-linked by peptides.

Cleavage, Competence and Pheromone: Recent publications have recognized how bacteria control their life cycle by the 13 two component signaling system and one ser/thr kinase and even the entry of donor DNA fragments if at all necessary[6,7]. We have recently observed that the diplococcic Streptococcus growth curve defines their physiological states of growth from birth to death in three phases, pre-competent, competent and post-competent. There is no good reason to think of any genotype difference between the same bacteria in different growth phases. However, cleavage formation at the mid cell position initiates the competent-phase with an ability to excrete pheromone (17aa peptide) to sense their immediate environment. In this phase they reproduce their own kind under the regulation of one or two -component signaling system. Even in an adverse environment when penicillin is added, the few pre-existing point mutants are selected. We think that the point mutants arising by the infidelity of replication are selected by our medicines. Nutrients released by the bactericidal effect of penicillin help these few mutants to overgrow and thus defeat our purpose. Briefly, target of penicillin is the penicillin binding proteins of high molecular weights: PBPs, 1a, 1b, 2a, 2b, 2x. Of them PBP 2x is highly important as S. pnemoniae with a point mutation in PBP 2x has not been isolated and we think bacterial reproduction is affected. The PBP 2x is an enzyme involved in the late stages of peptidoglycan assembly which is a requirement for bacterial growth and reproduction. Since PBP2x localizes to the bacterial reproduction site (cleavage), and probably it has a relationship with its PASTA domain[6,9]. In a few recent publications, it has been shown the StkP(Ser/Thr kinase) activation and substrate recognition depend on the presence of a peptidoglycan-binding domain comprising of PASTA(PBP proteins and Ser/Thr kinase associated domain) repeat. The StkP regulated in a growth dependent manner and likely senses intracellular peptidoglygan subunits present in the cell division septa (cleavage). We therefore strongly believe that StkP displacement should interfere in cell division. The cell wall thinning by xylitol phosphate affects cleavage and therefore the StkP and PBP2X.

Significance of Dr. Griffith's smooth and rough colonies deserves to be expanded because the competent diplococcic Gram-positive Streptococci reach their stationary or starvation- phase in solid blood agar media when the nutrients in growth medium are completely depleted[1]. Bacteria grow initially as smooth or small colonies but gradually become uneven, termed rough colonies after 24 hours or longer incubation. We have repeated his experiment also with S.oralis and S.mutans in addition to S.pneumoniae. Our scanning electron micrograph of this rough colony demonstrates clearly how these bacteria prevail in a latent phase with a high potential of pathogenesis. We must not forget the colony does not mean an individual diplococcus but a million together in their chain of inherent heterogeneity(Fig. 1). Recently, we have

recognized that in the complete growth cycle of diplococcic Streptococcus there are also pre-competent and post-competent phases in addition to their competent phase(Fig. 1). This observation has helped us to think of an alternative preventive treatment for the diseases caused by the diplococcic streptococci, S.mutans (dental) and mitis group pathogens. We have to accept that the antibiotics resistance crisis arises by selection of the point mutants pre-existing or arising in the growing bacterial population. In fact, recent publications in Streptococcal genetics have also ended up in an eclipse phase by reporting "that the donor DNA in a single stranded form with 3-prime OH end ends in an eclipse phase". Despite repeated attempts over the years we have failed to isolate the SS -DNA of any appreciable length from their eclipse-phase complex[13]. After the eclipse phase, the pathogens have altered their genetic character(s), antibiotic sensitivity patterns or antigenic variations but unfortunately investigators have assumed for a long time a homologous recombination has taken place between the donor DNA (3 prime OH end, ss) and the competent recipient chromosome in the cytoplasm!!

Our interpretation of Dr. Griffith's experiments as illustrated in text books differs : the heat killed extracts of smooth colonies when added to the rough colonies, the bacterial rough colony (a million contained in a chain) ruptures into their heterogeneous population and individual diplococcus starts re-growing(Fig. 2.). Their nutrition is obviously derived from heat killed extracts and they start new growth to gradually reach to saturation. Obviously, the potential of pathogenesis is still retained by the live diplococcic population. Regardless of their growth in solid blood agar or liquid nutrient broth, the stationary phase population is conditionally inert. Dr Griffith's rough colony is therefore a mixture of different phases of growth (shape, size and viability differ) and obviously the live ones are still capable of producing diseases[14]. In our recent work, we have also determined that the diplococcus S.oralis grows in –chains and their total population are stably contained in- chains if they all (100%) are grown in the presence of 5-carbon sugar alcohol xylitol[15,16,17]. In all clinical laboratories, the century old Gram-staining technique with a dye crystal violet has been extremely useful in distinguishing between the Gram -negative and Gram-positive classes of bacteria. The Gram-negative control E.coli K-12 appears pink and the Gram positive Staphylococcus appears purple. Based on such reference strains we have confirmed that the contours of diplococcic bacterial colonies satisfy their description as smooth and rough colonies. We want to think this is an index of natural transformation which represents diplococcic bacterial physiological states of growth, smooth (log-phase) and rough (stationary –phase) colonies on blood agar medium. Similarly in liquid broth we differentiate them, either in growth –phase or in stationary phase respectively[18]. Unlike E.coli K-12, these diplococcic Gram –positive bacteria keep growing in chains with the increasing lengths until their essential nutrients in the growth media are depleted, however phosphorylation of xylitol may not simultaneously stop which depends on the availability of ATP(ATP dependent fructose phosphotransferase system)[19].The pneumonia causing bacteria may live silently (latent phase) in their long chains. However, we must not forget that all their family members live in-chains with heterogeneity of sizes and shapes. Above all, the real old ones may only be visible as pinkish colonies or remain invisible even by Gram-staining technique because of their thinning of cell wall thickness[13].

Crystal violet is used as a dye but its interaction with the bacterial cell wall depends on bacterial cell wall thickness. Because peptidoglycan layers are unfolded when bacteria are grown in xylitol resulting in protoplasts and poorly stained and or not stained at all; but their existence in the chains of heterogeneous population are ruptured during dilutions in laboratories and therefore the majority of their population has been overlooked. We have demonstrated that these diplococcic Gram positive bacteria prevail in- chains with heterogeneity but all their family members can be contained by growing them in the presence of xylitol (2 – 5%). Recently we have shown the pre-competent phase of diplococcic streptococcus under normal growth condition without xylitol. Until now this pre-competent phase has not been microbiologically analyzed and therefore the investigators are limited by the incompetent and competent states. We also want to make it clear that there is a big difference between the pre-competent and the incompetent states of growth, young and the old respectively.

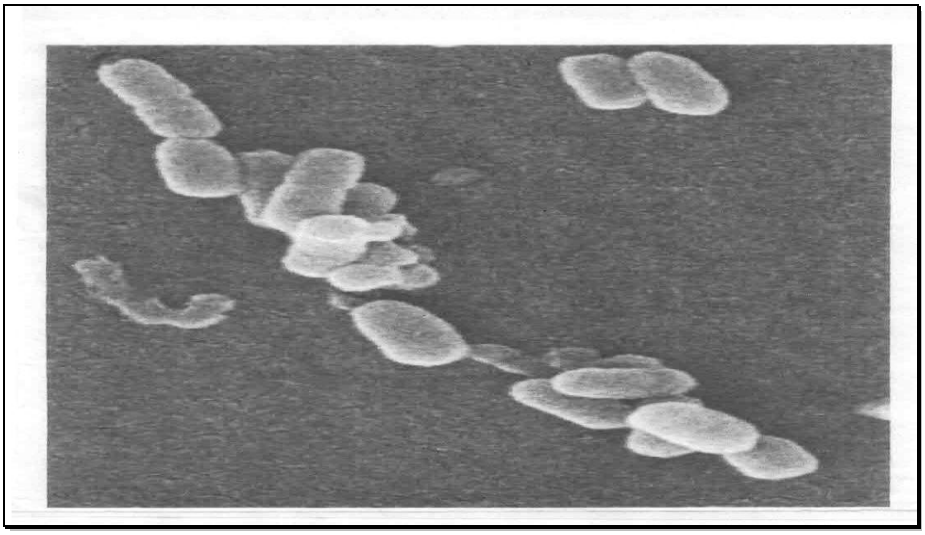

Fig. 2: Griffith's rough colony as visualized by scanning electron microscopy (SEM) at a magnification of 3000X. However, we have done this experiment with a genetically well characterized strain of S.oralis and culture started with a pure colony. Besides heterogeneous members of the rough colony in a long chain, individual diplococcus members in a pair (above this long chain) and the old population in a short chain (below the long chain) are also present.

These chains are partially ruptured during dilutions of the overnight cultures in our research laboratories because of the shearing force induced by pipetting or by suspending colonies in fresh nutrient broth. Therefore, their real heterogeneity has mostly been ignored (Fig. 2). The stationary phase culture, equivalent to rough colony when grown on blood agar (solid medium) is never 100% a virulent but the mixture of both virulrnt and avirulent. In the same field of electron micrograph, the diplococcic individuals in a pair and the old in a short chain are also seen. They are dissociated from the main chain probably during sample preparation. In the published articles we have seen the reproductive phase (competent/transparent/smooth) and the stationary phase (incompetent/opaque/rough) of these diplococcic bacteria[(18)].

Competent/Transparent/Smooth and Incompetent/Opaque/Rough Colonies

The competent population initiates their reproductive phase and continues until they reach a starvation phase or latent phase. Unlike E.coli K-12, this latent or stationary phase of diplococcic Streptococcus consists of heterogeneity of the competent and the incompetent bacterial cells but all appear to prevail in a single chain of unlimited length. Therefore the breakage of the chains by the laboratory dilution procedure into their individual members shows the purple colored diplococcic population as a minority by the standard Gram-staining procedure (Fig. 1). The remaining population in the same field of view is highly diminished in sizes (much smaller than 1.7 µm), poorly stained and many of them may even prevail as invisible because of their inability to adsorb crystal violet. We have observed a percentage of the population, spherical and highly diminished in size but light purple (pre-competent growth phase) before they are capable of producing pheromone and morphologically appear to be diplococcic.

That explains why in our Gram- stain preparation of overnight cultures diluted 10,000-fold in fresh broth, are not crowded at all[(7)]. Now we know that they prevail in a long chain with heterogeneity of their population and Shearing force introduced during dilution breaks the chain apart resulting in the release of all members. In support of our observation, we have also observed some pink colored clusters in addition to well defined purple colored individuals. The old with diminishing cell wall thickness are poorly stained or not stained at all. They appear pinkish but not like Gram negative E.coli K-12 and sizes are significantly diminished. Based on existing data, we want to agree that the competent bacteria initiate their new growth with the formation of a cleavage at the mid – point of their oval shape[(12)]. Based on all data available we strongly believe all competent, transparent and smooth colonies represent diplococcic Gram-positive bacterial growth phase (early, mid or late log phase). Opaque and rough colonies consist of heterogeneous population of diminishing sizes. We want to precaution all teaching laboratories that description of Pneumococcal colonies can't guarantee about their bacterial pathogenesis status because all the individual members contained in are in different phases of growth. We agree that the bacterial population in- chain is conditionally not virulent until they are ruptured by the stress induced.

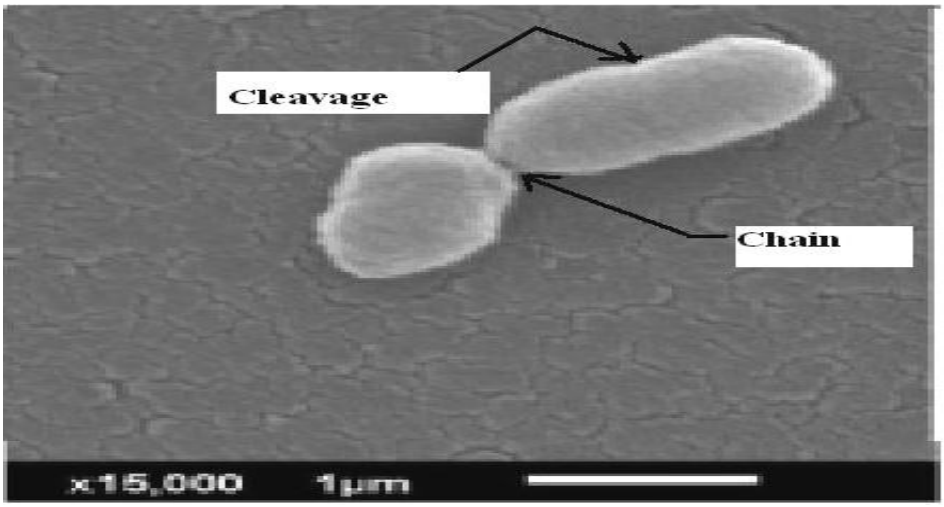

Fig. 3: The single oval shaped bacterium with a cleavage at the middle forms a diplococcic shape (magnification 15000X). Scanning electron micrograph of the two diplococcic shaped bacteria are linked in a chain. Diplococcus never means two but the cleavage formed at the middle of the oval shaped bacterium.

We strongly believe that the cleavage is an index of reproduction - phase which differs considerably from their stationary phase (latent phase). Bacterial cell division begins at the cleavage site, then it seems logical that the chromosome is attached to the bacterial cell membrane but probably at the cleavage site (our unpublished data). We speculate that DNA replication and segregation into S.pneumoniae daughters are simultaneous events. One or two-component signaling system (bio-communication) regulates the initiation of bacterial reproduction cycle and selection of PBP mutants already originated by the error-prone replication in the presence of penicillin. Such bio-communication is not anything new in microbial world but details may vary.

Based on our data presented in the recent International meeting (Saha Institute of Nuclear Physics, India, July 3, 2013), we like to predict that the same serine/ threonine kinase may be involved in allowing both Mycobacteria and Pneumococci to hide in human body, although they are not at all related by their chromosomal G/C content. Therefore interfering in these microbial bio-communications by the presence of xylitol or by other means we should be able to break their silent existence in the latent phase and make them grow in the presence of our available treatments and or develop a new approach. Antibiotics resistance crisis is real for both pneumonia and TB and therefore an alternative treatment at a low cost is highly desirable.

CONCLUSION

In the book "The PNEUMOCOCCUS, 2004; Edited by Elaine I. Tuomanen et al", the transformation in S.pneumoniae describes that the double stranded DNA fragment (s) (origin of such DNA fragment remains undefined), adheres to the competent recipient[10]. The fratricidal effect of the competent destroys the incompetent old population but we have not seen such violence among the bacteria belonging to the Mitis group (20)! Our published work has demonstrated that in the latent phase, the progeny remain clustered around the old incompetent parents but all are still in-chains, an ideal concept of joint family[7]. There is a thinning of cell wall thickness with the formation of protoplasts (our unpublished data on incompetent population separated by differential centrifugation and visualization) but lysis occurs if precautions are not taken to protect them. At the centre of the rough colony grown for 48 hours on blood agar medium (starvation), there is a little lysis but such a lysis is absent during 24 hours of growth. The nutrients released after 24 hours of growth are likely to cross-feed the competent ones present in the same colony, start afresh reproducing. The periphery of such colony looks very uneven and we call them rough colonies. We think that heterogeneity of population contained in the same bacterial chain is responsible for such uneven shape of the rough colony. In contrast we have never seen such uneven periphery with E.coli K-12 even after 48 hours of growth. The same cleavage accommodates StkP mediated two component signaling pathway and the PBP2X essential in diplococcic bacterial reproduction-phase. The question arises that before entry into the recipient, does the donor DNA fragment(s) at all adhere to the competent recipient's cell- wall? The mechanism by which the recipient allows the donor DNA fragment to enter and subsequent interaction with the homologous recipient chromosome need direct evidence. Such mutations may not require any double stranded DNA fragments to begin with but nucleotides with the single stranded 3-prime OH primer formed by the effect of EndA nuclease in the membrane ends in an eclipse phase! The RecA dependent homologous recombination as claimed on the basis of our experience in the laboratory experiments with Gram-negative E.coli K-12 is apparently absent in S.pneumoniae

(Palchaudhuri, S, unpublished). Despite all such academic effort, an intact long piece ss-donor DNA has so far not been isolated following the digestion of double stranded donor DNA fragments in the membrane by the EndA nuclease. The strand with 3-prime OH end appears but we think simultaneously it attaches to the membrane or integrates into the replicating DNA still in the membrane in collaboration with the available enzymes (RecA, Ssb etc). This observation provides an excellent reason that the eclipse phase and the origin of PBP point mutants of diplococcic streptococcus are not the two separate genetic events. Obviously, the SOS type repair of Gram-negative E.coli K-12 is absent in the S.pneumoniae because the SS-donor DNA never enters into the recipient cytoplasm. We should not forget that the activated RecA (SS –DNA and RecA complex*) formed in Gram-negative bacteria is absent in S.pneumoniae and so is their induced mutagenesis like the one reported in E.coli K-12 by Dr G. Walker. We speculate that in the absence of SOS repair in S.pneumoniae, the probability of induced mutagenesis is remote except point mutation of the PBP genes. Our work in progress will deliver a low-cost recipe for feeding our unavoidable enemy a low calorie sugar diet with fluoride before they wake up in their bed of human nasopharynx (Palchaudhuri S et al, 2013 and 2015).

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