Medical Biomaterials
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Lecture – 11
Biofilm

Hello everyone. Welcome to the course on Medical Biomaterials. We will continue on the topic of Biofilms. Biofilms as I said is most serious issue in the area of a biomaterial implants devices. Most of the biomaterials or implants fail because of formation of biofilms. And which reduces the life of implants or even devices. Now what is this biofilm? It is a population of microorganisms concentrated at a surface and it is surrounded by extracellular polymeric substance.

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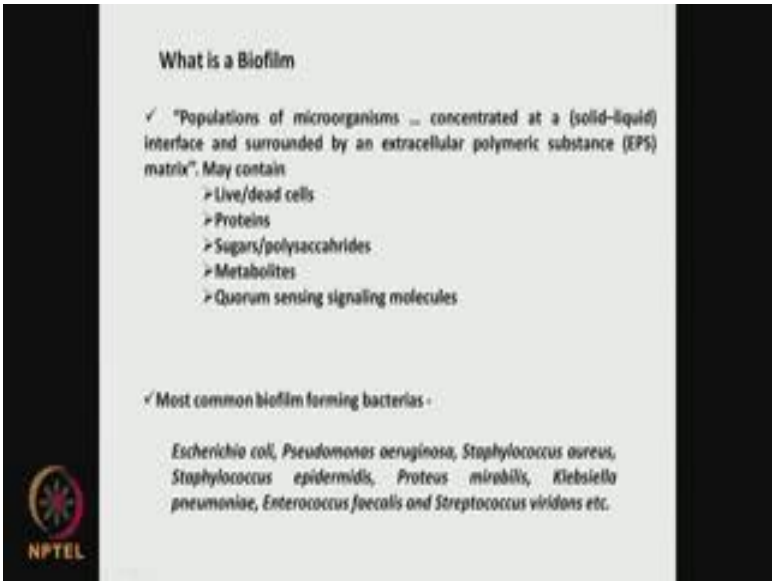

So, there is a matrix that is formed which sort of protects these bacteria and thereby they survive for a longer time, they are able to prevent getting killed by the antibiotics. So, some of them even develop antibiotic resistance and so on.

So, this biofilm may contain live or dead cells. It will contain proteins it will contains sugars polysaccharides metabolites even Quorum sensing signaling molecules, so most of the Gram positive Gram negative bacteria from biofilms. In various parts of a implants it could be a dental implant, it could be a cardiovascular stent, it could be ureteral stent, it could be a orthopedic material and so on; so E. coli pseudomonas staphylococcus aureus

staphylococcus epidermidis proteus mirabilis klebsiella enterococcus streptococcus so many different types of organisms that can form a biofilm.

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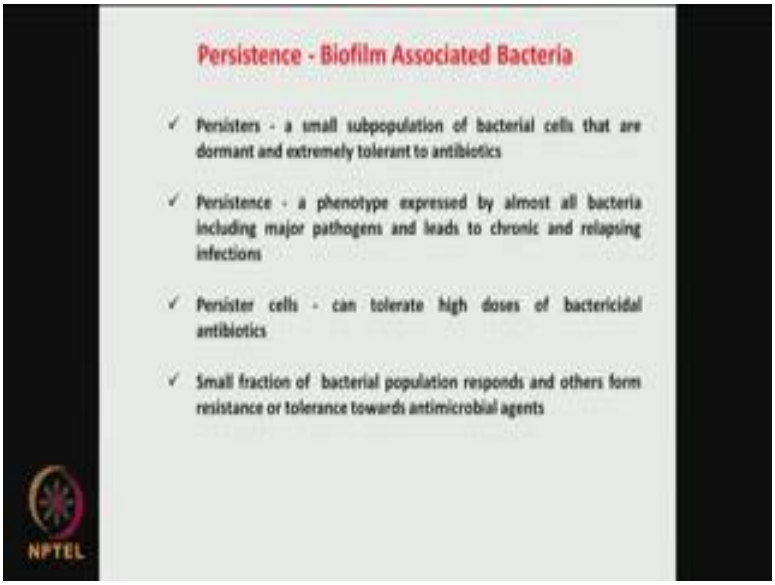

There is something called persistors. These are cells bacteria which are in biofilm and they start remaining for a longer time they become persistent. That is a small sub population of bacterial cells that will be dormant. So, they become extremely tolerant to antibiotics.

So, they acquire this antibiotic hence they persist in the biofilm. So, these are a phenotype expressed by all most all the bacteria and including major pathogen. This can lead to chronic and relapsing infection. So, because the biomaterial has been placed some bacteria survive for a longer time and they become immune to antibiotics also that is persister cells. They can tolerate high doses of bactericidal antibiotics. Small fraction of bacterial population responds and others form resistance or tolerance towards these antimicrobial agents.

So, why do they become they change their phenotype, there is an exopolysaccharide matrix that formed on the surface, it prevents diffusion of the antibiotics right to the bottom of the biofilm. So, many reasons happen we will talk about some of those reasons as we scroll down the class.

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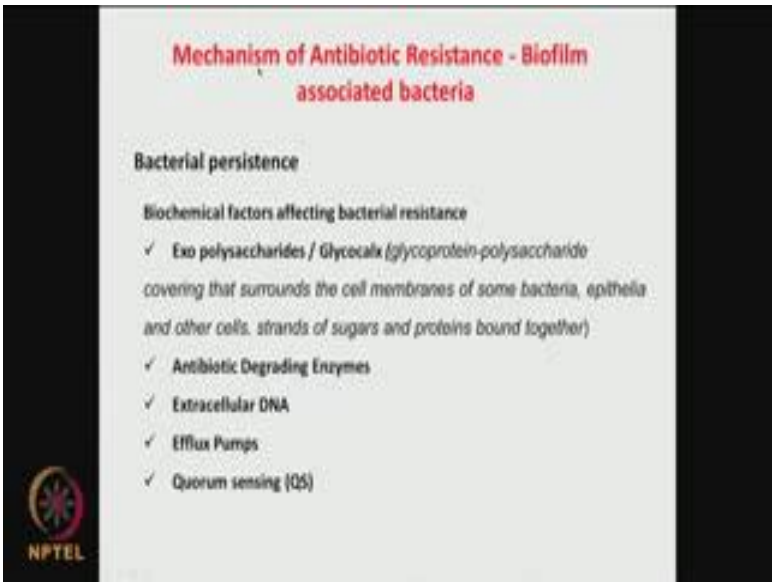

So, why do they get antibiotic resistance? This is called biofilm associated antibiotic resistance. There are many factors which are which lead them to have bacterial resistance. For example, the presence of exopolysaccharides and glycocalax; this glycocalax is nothing, but is a glycoprotein polysaccharide covering that surround the cell membranes of some bacteria. And even cells this is nothing, but strands of sugars and proteins bound together. So, it prevents the diffusion of the antibiotics. There are enzymes which are produced by the bacteria.

And they are called antibiotic degrading enzymes. So, these enzymes as soon as the antibiotic is released into the system, they come and degrade the antibiotics. So, the antibiotics are no more effective, the extra cellular DNA which also affects which leads to antibiotic resistance. There are something called efflux pumps, many bacteria possess this particular protein called efflux pump. And these pumps out whatever foreign material that enters the bacteria, it could be an anti-microbial agent it could be an antibiotic, it could be a dye it could be a toxic chemical.

So, these efflux pumps throw them out thereby it prevents the killing of the bacteria. Quorum sensing, many bacterial species release a chemical called a Quorum sensing molecule it is a metabolized and just to inform each other that there is a certain cell population density. Quorum sensing helps in the biofilm formation, which basically

Quorum sensing molecules help the bacterial population; that means it has reached a threshold population they can form a colony.

So, all these factors have an influence on the bacterial species gaining antibiotic resistance. And we will look at each one of them little bit and this leads to biofilm associated infection also so, in biomaterial. So, the factors that are affect bacterial resistance we can group into biochemical factors, we can in molecular mechanism we can call it altered host factors. Biochemical factors like I mentioned exopolysaccharides that are produced antibiotic degrading enzymes that are produced extracellular DNA efflux pumps Quorum sense.

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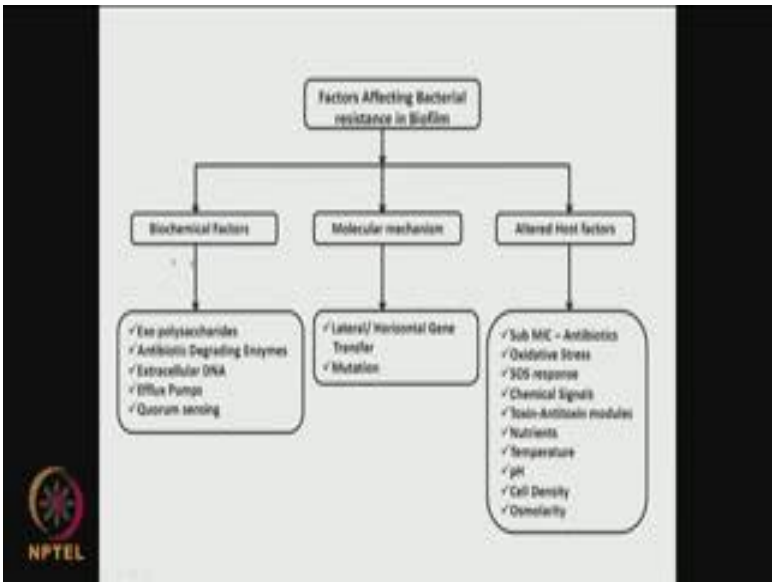

If you look at molecular mechanism, there could be a gene transfer, lateral gene transfers or horizontal gene, transfer sometimes the bacteria as soon as they settle on the surface mutates. All these can lead into resistance antibiotic resistance.

The third could be altered host factors. Because the antibiotics could be under some MIC concentration like I said the antibiotic has to diffuse through the biofilm matrix. So, the concentration of the antibiotics in the interiors or the biofilm could be some MIC. Oxidative stress, the oxygen diffusion also is hindered in the biofilm because of the presence of the EPS. So, many of the bacteria can be undergoing oxidative stress. SOS response, chemical signals toxin antitoxin modules that are produced nutrients amount of nutrients available inside the biofilm could be much less. So, they are under starvation

condition. Temperature may be affecting pH may be affecting even the cell density, and the finally the osmolality. So, all these are called the host factors which may influence the bacteria to gain resistance in the biofilm.

So, one needs to understand all these factors, and hence design surfaces which can prevent a biofilm formation as well as which will eradicate persistent cells.

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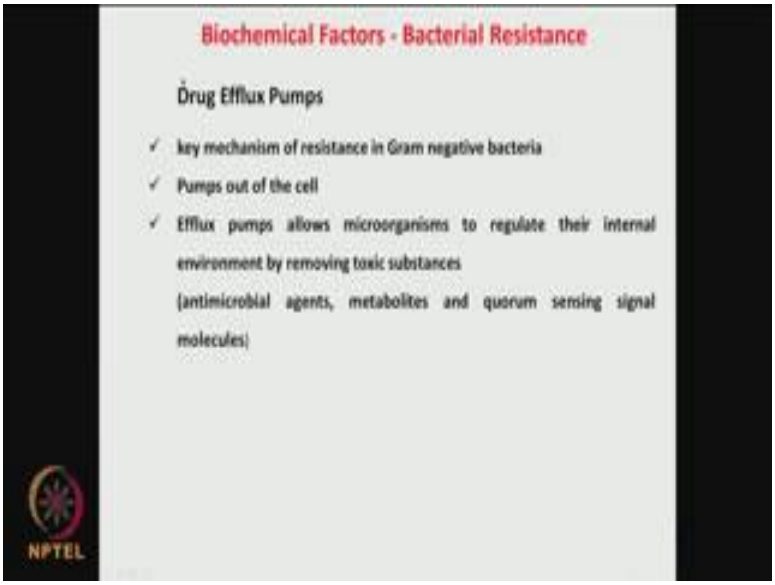

So, let us look at some of these factors the drug efflux pumps. Like I said these are large proteins which throws out whatever foreign material is inside. So, the pump out of the cell mostly they have found in Gram negative bacteria, they allow help the microorganism to regulate their internal environment by removing toxic substances, it can be microbial agent's metabolites whatever it is you know. These are called efflux pumps. There are many families of a efflux pumps you do not have to get very varied about these biological names, but you have to understand there are many families of efflux pumps.

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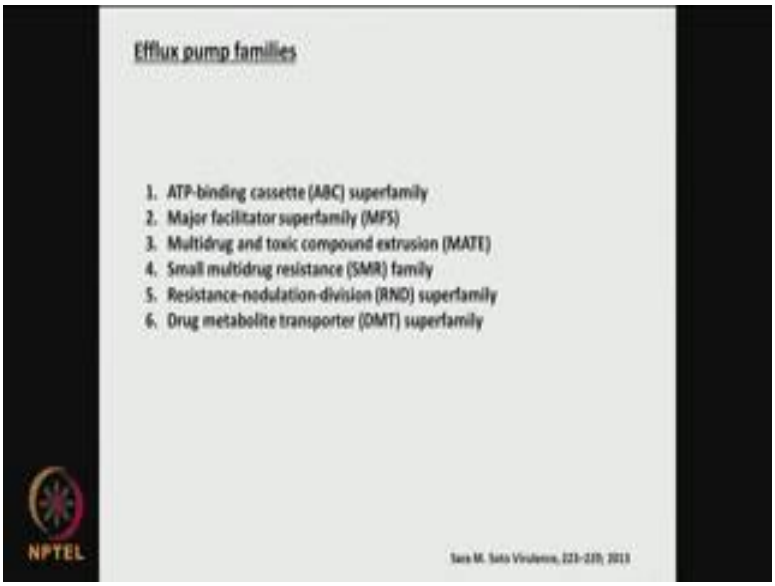

So, sometimes in the drug discovery if they decide to design molecules for one set family of a efflux pump inhibitor you could have several efflux pump families, which we need to one is called ATP binding cassette, superfamily other is major facilitator superfamily. There is a multidrug and toxic compound extrusion family, small multidrug resistance family, resistance nodulation division superfamily, drug metabolite transporter superfamily. So, even metabolites that are formed when the drug is introduced there are efflux pumps which can throw it out.

So, a large number of families are there these are called efflux pump families which throw out any foreign metabolite that is present inside the bacteria.

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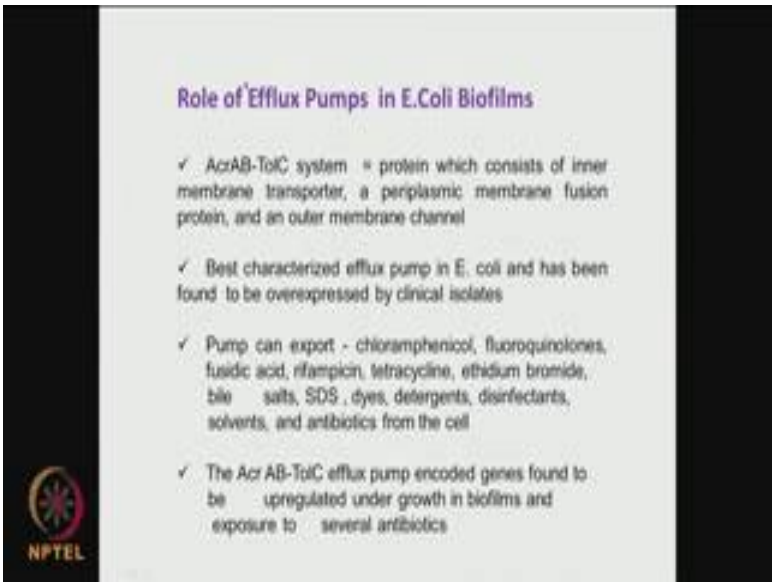

If you look at E. coli, E. coli has very well developed efflux pumps. This is a particular system a C r a b t o I c. This is a protein which consists of inner membrane transporter a periplasmic membrane fusion protein and an outer membrane channel. So, in the E. coli these transporters ride from the inside of the microorganisms. And they can throw the metabolite ride outside into extracellular.

These are best characterized efflux pumps in E. coli, and have been found to be overexpressed especially in clinical isolates, because clinical isolates developed lot of drug resistance. The pump can export as you can see antibiotics like chloramphenicol fluoroquinolones fusidic acid rifampicin tetracycline ethidium bromide bile salts SDS it is a fact like SDS dyes detergents disinfectants solvents antibiotics, large number of compounds can be thrown out, by this particular family. This family encodes genes found to be up regulated under growth in biofilms and exposure to several antibiotics.

So, in the biofilm as well as when the E. coli is exposed to antibiotics, these genes which encode this particular efflux pumps get up regulated. So, they are very active and they are found in especially in clinical isolates. So, there are efflux pumps inhibitors, their substances that inhibit the flux of substances mediated by efflux pumps. So, what we do is if we have efflux pump inhibitors, it can go and bind to those proteins and make the efflux pumps inactive then the bacteria become.

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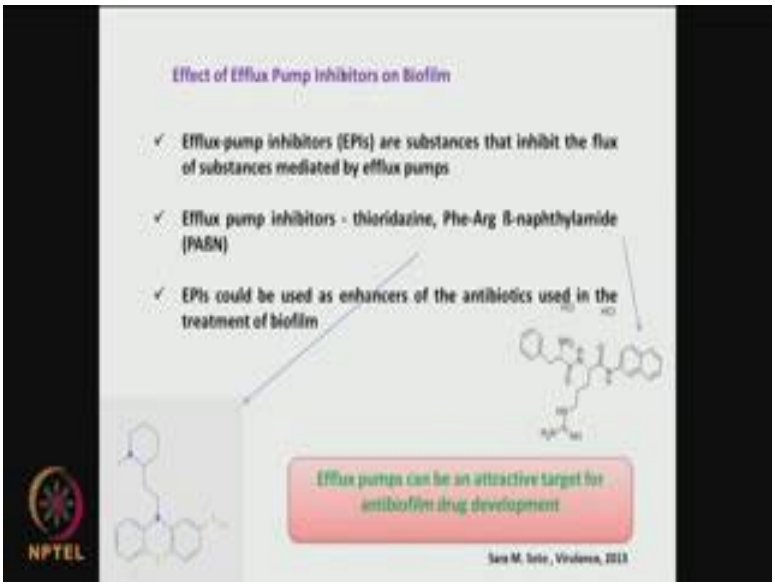

Under a any antibiotic it can be easily treated under any antibiotic and they become very sensitive to those antibiotics. So, one strategy of dealing this type of resistance is to give the efflux pump inhibitor as well as you give the antibiotic as well. So, they are many inhibitors thioridazine and then PA beta N. These inhibitors can have used as enther enhancer of the antibiotics in the treatment of biofilm. So, for example, this particular compound contains this type of hetero cycle with nitrogen and Sulphur. This is a compound which has got a arginine, naphthalimide here.

So, in addition to give an antibiotic, you can also give this one of this efflux pump inhibitors so that the biofilm can be eradicated.

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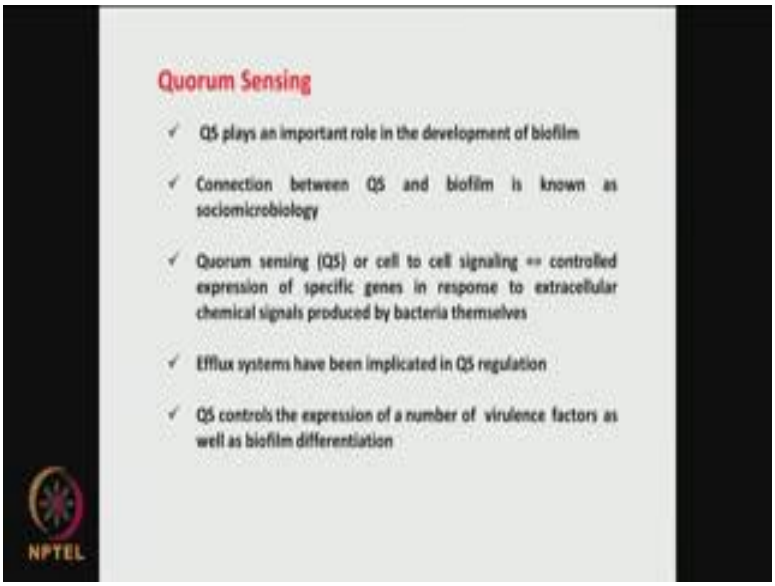

The next important activity in the bacterial biofilm is the Quorum sensing. Now this Quorum sensing plays a very important role in the development of biofilm. So, what is this Quorum sensing? When the bacterial population is very small and as the population keeps increasing they start releasing a metabolite that is called a Quorum sensing molecule. And when the concentration of this Quorum sensing molecule increases in the bacteria also realizes that their population has reached a threshold value and they start forming biofilm on a surface. So, Quorum sensing is almost like a signaling and the signaling helps for these bacteria to change their phenotype from the sessile form to the fixed form. So, there is lot of changes that happen as soon as they start settling down and form biofilm.

So, there is a very strong connection between Quorum sensing signaling and biofilm formation. This is almost like a social behavior of the bacteria. So, this Quorum sensing is a cell to cell signaling. And this is controlled expression of specific genes in response to this extracellular chemical signals produced by bacteria themselves. And also the efflux systems have been implicated in Quorum sensing regulation. So, the Quorum sensing not only changes the phenotype of the bacteria from the sessile or mob motile form into fixed or biofilm forming form, but it also affects the efflux pumps. It also controls the expression of number of virulence factors as well as biofilm differentiation.

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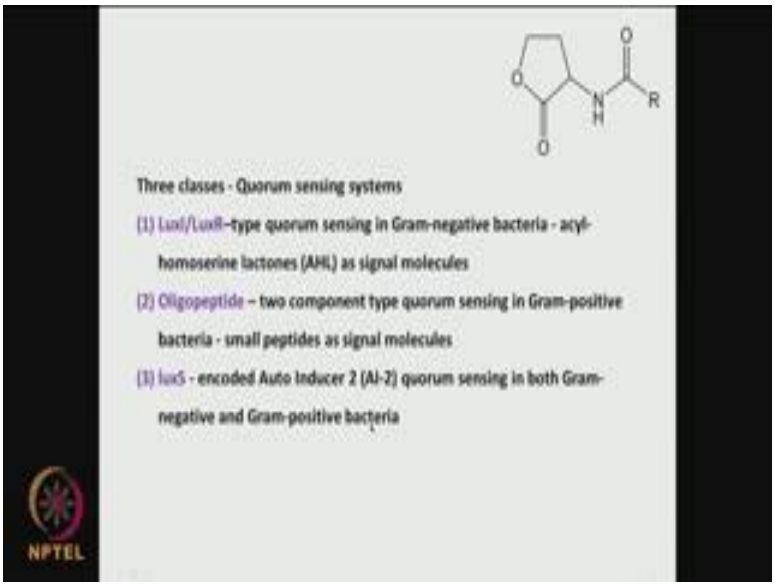

So, there are 3 classes of Quorum sensing systems have been reported actually. The first one is related to Gram negative bacteria. There is there are 2 proteins lux and LuxI and LuxR these are Quorum sensing in Gram negative bacteria. And the Quorum sensing signal in molecule is called acyl homoserine lactones which are the structure of these acyle homo serine lactones. So, there are lot of these type of functional groups, this lactone ring is there and there could be longer chains. So, you can have hydrophobic molecules are very short hydrophilic. These are all called signaling molecules. These are produced by the Gram negative bacteria. And at certain threshold concentration the phenotype of these bacteria changes because they realize their population density has reached a threshold value.

If you look at Gram positive bacteria they produced some Oligopeptides, these are 2 component types Quorum sensing in Gram sensing bacteria. These are small peptides equivalent this AHL signaling molecules produced for a Gram negative Gram positive produced a small Oligopeptides. Then we also have LuxS these are encoded auto inducer 2 Quorum sensing in both Gram negative and Gram positive bacteria. So, predominantly Gram negative produces this acyl homoserine lactones, Gram positives produces the oligipeptides. So, most of the percentage of bacteria is covered by these and of course, you also have some LuxS which is produced it is an auto inducer produced by both the Gram negative and Gram positive.

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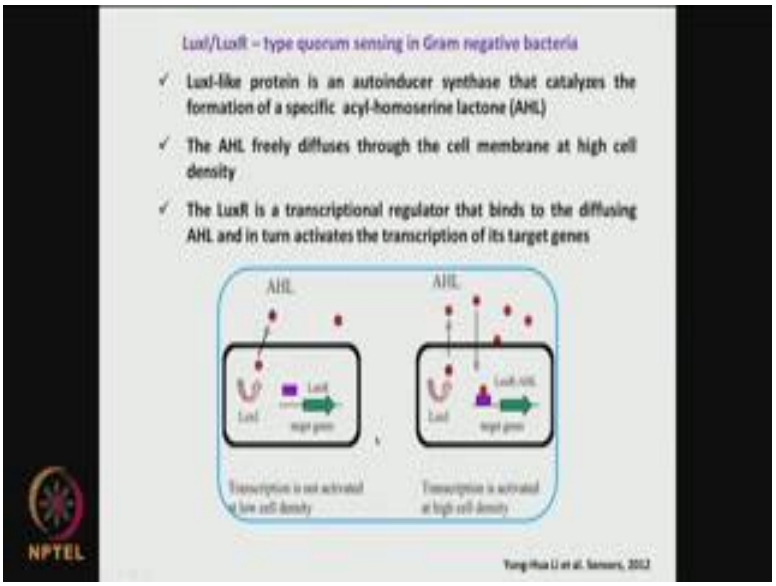

Let us look at how this LuxI and LuxR type of Quorum sensing in Gram negative happen. So, LuxI like protein is an autoinduser synthase that catalyzes the formation of a specific acyl homoserine lactone. So, the AHL is produced, it goes to the extracellular space. So, at low concentrations the AHL gets dispersed it diffuses freely through the cell membrane at high cell density. So, at high cell density they come back again and they bind to the LuxR. The LuxR is a transcriptional regulator that binds to the diffusing AHL back and in turn activates the transcription of it is target genes.

So, at lower cell density AHL also produced it diffuses out into the extracellular space and lost, but at high cell density the concentration of AHL is So, much some of it diffuses back and binds to LuxR which leads as soon as they binds this leads to lot of transcriptional regulatant that happens like moving of the bacteria from sessile to fixed or biofilm forming form, production of virulent factors activation of a efflux pumps and so on actually. So, in Gram negative bacteria this LuxI LuxR play very important role and the AHL the signaling molecule gets diffused out and then again it comes back and binds to the LuxR. This is how the action of AHL happens in Gram negative bacteria.

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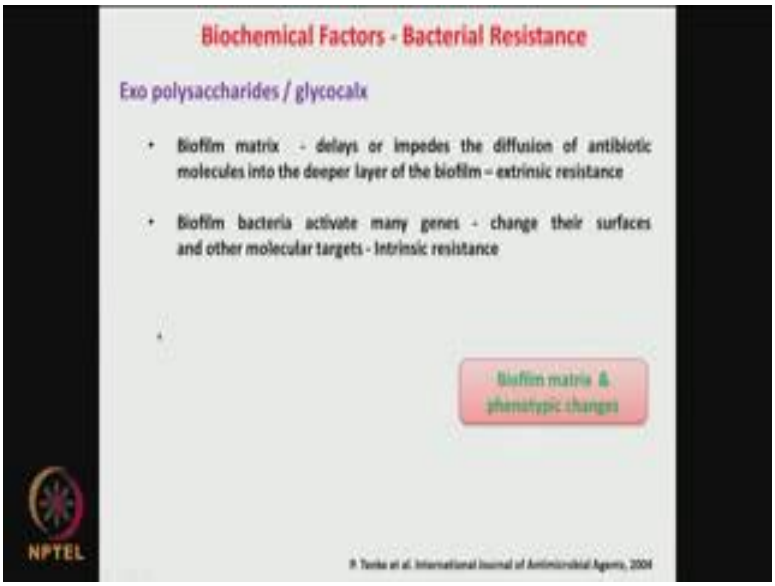

What are the other biochemical factors which lead to bacterial resistance? You have the exo polysaccharides and glycocalx. These are sugars and protein combination. So, they form a thick matrix on top of the bacteria. So, it delays or impedes the diffusion of antibiotic molecules into the deeper layer of the biofilm. So, they are more like physical effects prevention. Then biofilm bacteria they also activate many genes. So, their surfaces and other molecular targets also change.

So, the antibiotics are not able to go and attach, because their surface in molecular targets has changed. Whereas, the matrix the thick matrix prevents the diffusion of the antibiotic, that is that we call it as extrinsic resistance whereas, the changes on the surface and we can call it intrinsic resistance.

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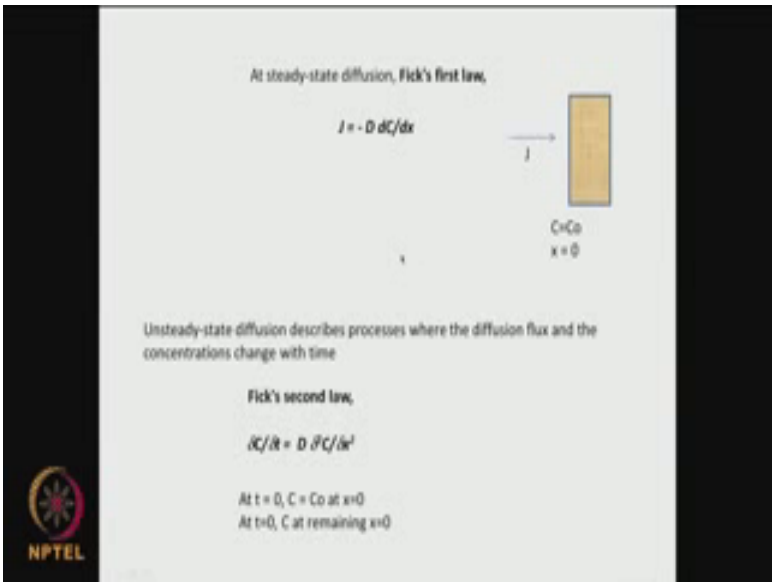

Now, if you look at it from an engineering point of view, the diffusion of antibiotics through from engineering point of view. We can see the antibiotic or nutrient or oxygen diffuses through the biofilm from one end and reaches right up to the bottom. So, we can here use ficks first law. You must have studied that long time back in your first year undergraduate what is this ficks. First law flux is equal to at steady state flux is equal to minus D that is diffusion coefficient d C by d x, C is the concentration x is the distance. So, d C by d x it is a concentration gradient and j is your flux D is the diffusion coefficient why this minus sign concentration of the antibiotic or nutrient could be is higher at the surface, whereas the concentration keeps decreasing.

So, if you have a constant flux we can assume this as a linearly decreasing concentration as we go deeper and deeper into the biofilm matrix. So, what does that mean? So, at the surface if the concentration of the antibiotic is C naught as we go down this concentration will be going down linearly, and it will keep going down and down. So, at the surface the concentration could be MIC, whereas if you travel down it may be coming much lower. And d sort of determines the slope of this linear line. So, if the d is very poor d is very small. So, the fall in concentration as we go along inside could be very low; that means, the concentration inside could be much lower when compared to the concentration at this surface of the biofilm.

So, this d diffusion coefficient is based on many factors how thick the biofilm is what is the morphology of the biofilm how much of exopolysaccharides that are produced. So, the diffusion coefficients can become very small. So, when it becomes very small the concentration inside will be much lower than the concentration that is found on the surface. So, this is a very useful relationship to keep in mind which tells you the mechanism or the reason why there is a huge fall in the concentration of the antibiotic as you travel inside this biofilm.

Now, this is at steady state, you can also have an unsteady state diffusion where the diffusion flux and the concentrations change with time also. So, we need to bring in the factor of time here. So, once we bring in factor of time here it becomes partial differential equation delta C by delta t, t is your time is equal to d d square C by d x square. So, we are considering only one-dimension x. So, if you can have y z you can put in d square C plus d y square d square C by d z square and so on. So, it will be this is a partial differential equation. That is why you put like this whereas, here we put like this. This is a ordinary differential equation, whereas this is the partial differential equation.

So, the concentration is a function of time as well as the distance. Here it could be just x, but we can have x y z all the 3 coordinates also. So, here at time equal to 0 C is equal to C naught at x equal to 0, and the surface at time equal to 0 concentrations in the remaining; that means, inside the biofilm will be 0. So, at time equal to 0 concentrations side the biofilm is 0. So, we concentration of the antibiotic or any metabolite at the surface could be C naught. So, if this is it is possible to solve this equation also. It is not good too much into that and makes you worried, but it can be solved and you can have relationship with that describes C as a function of time as well as x.

Whereas, in the first law it is quite simple when we have the constant flux j on the left hand side then d C by d x looks like a linearly decreasing concentration as we move along inside the biofilm. Now we can have different situations for the flux and we can have different types of graphical observations let us look at it.

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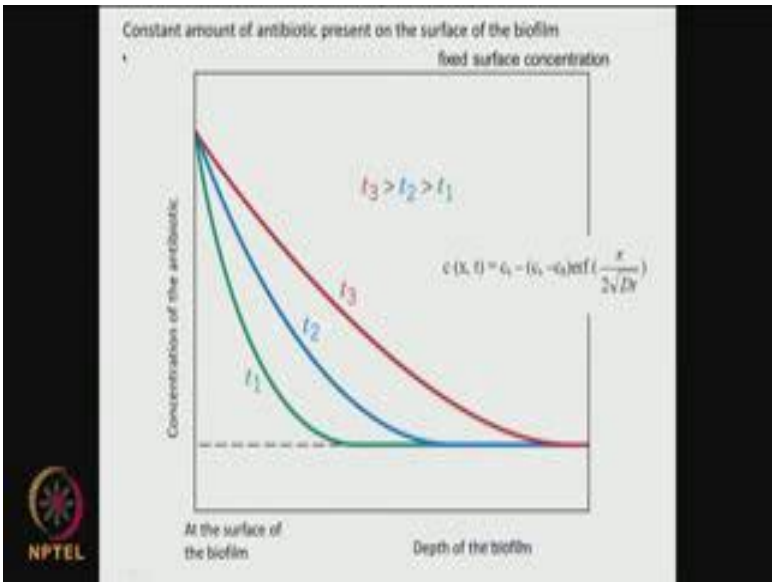

Suppose, we have fixed surface concentration that is constant amount of antibiotic present on the surface of the biofilm there is fixed surface concentration. So, at the surface of the biofilm concentration of the antibiotics could be this much, but as you go inside the depth concentration may be falling at a particular time t 1. Then as a time keeps going up it may slowly building up like this t 2 t 3 and so on actually like this. And how fast it increases that depends upon the diffusion coefficient and the depth of the biofilms and so on actually.

So, if you have constant amount of antibiotic present on the surface; that means, the antibiotic is not depleting constant amount. So, at the surface concentration will be. So, much as you go inside the biofilm the x axis is depth of the biofilm the concentration will fall down like this, but as you increase the time it will slowly build up like this; that means, t 1 t 3 is greater than t 2 greater than t 1. Now it is mathematically represented in this form concentration is a function of x and t C s is the concentration of the surface surfaces. And this is how it is given the relationship is given like this where d is the deficient coefficient t is the time x is the depth.

Now, you can have another situation where certain amount of antibiotics is placed on the surface of the biofilms at time equal to 0 that is fixed amount of dopant; that means, the concentration at the surface also will decrease because as it keeps diffusing in. So, at very low time the graph will look like this as a function of depth.

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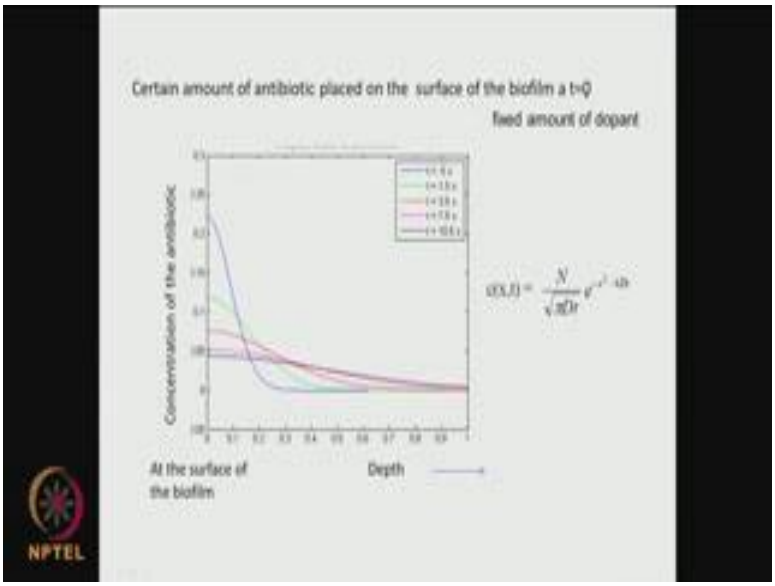

So, initially at the surface we will have certain concentration as you go inside concentration will drop dramatically, but as time progresses the concentration inside may increase, but the concentration at the surface will fall down because we have fixed amount of antibiotics as time progresses we are going to have like this. So, at the surface also it will go down inside will slightly go up and it is mathematically given by this particular relationship. So, we have 2 types of situations where we have fixed surface concentration that is constant amount of antibiotic present and we could have only some amount of antibiotic is placed on the surface. So, that gets also depleted as the antibiotic starts diffusing in. So, we have a graph as shown like this whereas, in the other class the concentration versus distance keeps going up and up with increasing time.

So, as you can see from these explanations that the antibiotic amount inside the biofilm will be much lower than what is present in the surface. So, if the concentration at the surface is constant, then there could be a slow increase in the antibiotic inside the biofilm also, but if the concentration at the surface is also depleting, then we are not going to have a slow increase in antibiotic amount inside the surface. So, the bacteria inside the biofilm are always exposed to very low concentrations of antibiotic which could be below it is MIC hence bacteria start acquiring antibiotic resistance.

So, this is your physical phenomena which are based on the diffusions, through a surface. And this can be explained both by using ficks first law as well as ficks second law. Once

more as you can see ficks first law tells you the flux that is on the left hand side is equal to minus d diffusion coefficient d C by d x, where C is the concentration x is the distance. So, it is a linearly decreasing concentration as a function of time, if the if the left hand side is constant. So, at the surface concentration is high. So, it keeps going down, but if you look at the ficks second law it brings in time as well into it into the picture.

So, we have C the concentration function of time as well as the distance. So, we use partial derivatives here, d C by d t is equal to diffusion coefficients d square C by d x square. If you have the wide axis also then you will have d square C by d y square, if you observed then it is d square C by d z square. So, this is the ficks second law which tells you the concentration can vary as a function of distance or as you travel inside the biofilm as well as a function of time as well. And from these laws we can develop certain pictorial visualization of how the antibiotic concentration could be distributed inside the distance as a function of distance as well as a function of time.

So, in first situation we have fixed surface concentration that is constant amount of antibiotic flowing across the biofilm. So, at the surface concentration of the antibiotic could be very high, it falls down dramatically as you go inside the biofilm, this is at one particular time as a time increases this graph starts moving up and up and up and up and ultimately the concentration inside could also be very high. That is when the antibiotic amount present on the surface is also constant concentration. And this is expressed by this particular mathematical relationship. Whereas, in the other situation we have constant amount of antibiotics placed on the surface and after that we do not replenish the antibiotics.

So, it drops dramatically as you go down in distance, but because the antibiotic also starts diffusing the cons the concentration of antibiotic on the surface also keeps going down. So, at one time you will have a group like this, as a time increases the graph may become like this and like this and like this and so on. So, the antibiotic is of no use as you can see beyond a small distance and with as you go travel inside you have very low concentrations which may lead to antibiotic resistance because the bacteria is exposed to sub m n C concentration of the antibiotic.

And generally when a patient after a implant surgery is given antibiotics we can assume this type of scenario, where initially the surface has large amount of antibiotics and afterwards the surface concentration of antibiotics also keeps going down. So, in the interstices or in the depths the concentration of antibiotic is below sub MIC.

So, we will continue further on this biofilm and effect of biofilm on implants.

Thank you very much for your time.