博 士(工 学) メイ ティティワット

学 位 論 文 題 名

Factors controlling development and maturation of *Escherichia coli* biofilms

(大腸菌バイオフィルムの発達と成熟に関わる制御因子)

学位論文内容の要旨

Biofilms have distinct morphological and biochemical properties that distinguish them from free-living planktonic cells. Bacterial biofilms cause serious health and economic problems when they are found on medical and industrial devices referring to antimicrobial agents resistance. Microscopic observation, predominantly of Gram-negative bacteria, has led to a general description of biofilm formation as a temporal process involving transition through distinct stages of multicellular organization. In addition to morphological descriptions of biofilm formation, there is also increasing interest in the conjugative gene transfer and the global gene expression that control biofilm formation. Therefore, in this study, we investigated the biofilm development and maturation by *Escherichia coli* (*E. coli*) harboring the conjugative plasmid, and its capacity to resist various antimicrobial agents. Furthermore, the minimal gene set, which is required for biofilm formation, was also described to understand the metabolic pathway and its regulatory network inside biofilm cells.

(I) The important of conjugative F plasmid during *E. coli* biofilm formation, it has been shown that *E. coli* harboring the de-repressed IncFI and IncFII conjugative F plasmids form complex mature biofilms by using their constituent F pili connection, whereas a plasmid-free strain forms only patchy biofilms. In this study, we, therefore, investigated the contribution of a natural IncF conjugative F plasmid in formation of *E. coli* biofilms. Unlike a de-repressed F plasmid, the presence of a natural IncF F plasmid promoted biofilm formation by generating the cell-to-cell mating F pili between F+ cell pairs (approximately 2-4 pili per cell) and by stimulating the formation of colanic acid and curli meshwork. The formation of colanic acid and curli was required after the initial deposition of F pili connection to generate a mushroom-type biofilm structure. In addition, we demonstrated that the conjugative-factor of F plasmid, rather than pilus synthesis function, was involved in curli production during the biofilm formation, which promoted cell-to-surface interaction. The presence of curli played an important role in the maturation process. The microarray experiments were performed to identify the genes involved in the curli biosynthesis and regulation. The results suggested that a natural F plasmid was more likely an external activator that indirectly promotes the curli production via bacterial regulatory systems (the EnvZ/OmpR two-component regulators and the RpoS and HN-S global regulators). These data provided new insights into the role of a natural F plasmid during the development of *E. coli* biofilms.

(II) F plasmid-mediated F+ × F+ mating during *E. coli* biofilm formation, the ecological role of plasmid in sessile bacteria has been largely overlooked and reported, since the conjugative plasmids directly induce biofilm formation through the expression of conjugative pili. However, the mechanistic role of these factors during biofilm formation has not been determined in molecular detail. Consequently, the objective of this work was to elucidate the contribution of F plasmid on global gene expression inside *E. coli* biofilm cells. By using reverse transcription quantitative PCR, we showed that the pilus synthesis and mating-aggregate stability genes were highly expressed, but the DNA transfer, conjugation control and surface exclusion activities decreased to undetectable levels. The non-conjugative factors were involved in the production of curli and colonic acids. The high mating efficiency and long pili were observed by electron microscope, indicating F+ × F+ mating, so called F- phenocopies phenomenon. In addition, the F- phenocopies were generated to compare the gene expression patterns with *E. coli* biofilm using DNA microarray. The results showed that the biofilm lifestyle, although sharing similarities with F-phenocopies, triggered the expression of specific sets of genes as well as curli and colonic acid biosynthesis genes. We concluded that *E. coli* biofilm maturation is a self-assembly process composed of F- phenocopies sub-

populations requiring only effective cell-to-cell adhesion. In this case, the pili and mating-aggregate stability of F-phenocopies play an important role in cell-to-cell interaction. The curli and colanic acids promote and maintain a complex 3D structure of mature biofilms.

(III) F plasmid-mediated TraMJ signaling during *E. coli* biofilm formation, the F plasmid of *E. coli* allows horizontal DNA transfer between an F+ donor cell and an F- recipient. Expression of the pilus genes is tightly controlled by a number of factors, including the following plasmid-encoded regulatory proteins: TraJ, and the autoregulators TraM. However, the unusual expression of F pili between two F+ cells (F- phenocopies) has been observed during the development of *E. coli* biofilm. The F+ × F+ mating was resulted from the secondary characteristics of stationary phase-like sessile bacterial population during the formation of microcolonies. Here, we found that traM and traJ genes were up-regulated in microcolony biofilm, and later promoted the development of microcolony to mature biofilm. We then demonstrated that the interaction between traM and traJ involved in the F+ × F+ piliation. The localization of TraMJ expression was found on the substratum inside microcolonies indicated that F pili are the initial cell-to-cell adhesion. We showed that TraMJ signal were quorum sensing-like molecule. TraM and TraJ were secreted and assembled outside bacterial cells. In addition, the interaction between TraMJ was regulated by H-NS from the host cell, and each molecule could be produced from different cells. These indicated the role of F transfer in adaptive physiology in starved or stationary-phase cells during biofilm development.

(IV) Minimal genes set requirement for *E. coli* biofilm formation, the concept of integrated high-throughput experiments and computational data analysis of the genome-scale metabolic network is a promising approach to study cellular functions based on the interaction of the cellular components. Here we reconstructed a novel integrated genome-scale network and minimal gene set of a transcriptional regulatory and metabolic connection during biofilm formation. The commonly regulated genes have also been identified using the temporal gene-expression analysis guided by metabolic pathway structure. In addition, we introduced an alternative genetic strategy for searching either the most important genome-wide profiling of biofilm formation or biofilm-specific regulation using the engineered reduced-genome *E. coli* strains. Biofilm formation ability of both large- and medium-deletion strains (2.4-29.7% and 0.1-2.0% genome reduction, respectively) were investigated, and the temporal gene expression analysis was performed for the most genome-reduced biofilm-formable *E. coli* strain (17.6% genome reduction) during the biofilm formation. The minimal gene set for developing a 3D biofilm structure was obtained, indicating that at least the effective auto-aggregative factors and its regulators must be required during the development and maturation of the biofilm. Different kind of amino acids was sequentially secreted in each developmental stage wherein triggering a modification of growth conditions within biofilms. This integrated approach is able not only to predict the outcomes of growth phenotypes and temporal gene-expression experiments, but also to indicate knowledge gaps and identify unknown components and interactions during the biofilm formation.

(V) Antibiotic resistance during *E. coli* biofilm formation, biofilms gain resistance to various antimicrobial agents at the expense of other protective phenotypes, and the presence of antibiotic resistance genes is thought to contribute to a biofilm-mediated antibiotic resistance. Here we showed the interplay between the tetracycline resistance efflux pumps TetA(C) and the ampicillin resistance gene (bla) in biofilms of *E. coli* harboring pBR322 in the presence of the mixture of ampicillin and tetracycline. *E. coli* in the biofilms could obtain the high-level resistance to ampicillin, tetracycline, penicillin, erythromycin and chloramphenicol during biofilm development and maturation, as a result of the interplay between the marker genes on the plasmids, the increase of plasmid copy number, and consequently the induction of the efflux systems on the bacterial chromosome especially the EmrY/K and EvgA/S pumps. In addition, we characterized the over-expression of TetA(C) pumps that contributed to osmotic stress response and were involved in the induction of capsular colanic acid production, promoting formation of mature biofilms. However, this investigated phenomenon was highly dependent on the addition of the subinhibitory concentrations of antibiotic mixture, and the biofilm resistance behavior was limited to aminoglycoside antibiotics due to the unbalanced influx in biofilm cells. Thus, marker genes on plasmids played an important role in both resistance of biofilm cells to antibiotics and in formation of mature biofilms as they could trigger specific chromosomal resistant mechanisms to confer a high-level resistance during biofilm formation.

In summary, the mating F pili between pairs of F+ cells of *E. coli* are required for formation of the 3D mushroom-type biofilms as they stimulate colanic acid and curli production. These findings provide a basis for the connection between natural conjugative gene transfer and biofilm formation. We also succeeded to identify the minimal genes set for biofilm formation using the engineered reduced-genome *E. coli* in combination with temporal DNA microarray and in-silico analysis. This approach provides the alternative strategy to look for the important genes in each biofilm developmental process, as well as to reveal the minimal genotypic overview of biofilm biology. Finally we presented that *E. coli* biofilm could gain high-level resistance to various antimicrobial agents by stimulating efflux pump systems. These secondary phenotypes stimulated high osmotic pressure and then directly

promoted the progressive development and maturation of biofilms, which makes the biofilm more difficult to treat. Therefore, the handing of antibiotics must be very careful, and the spread of antibiotics to the environment should be more concerned.

学位論文審査の要旨

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(大腸菌バイオフィルムの発達と成熟に関わる制御因子)

細菌は多くの場合、固体表面に付着し“バイオフィルム”を形成して存在する。バイオフィルムとして存在している細菌は、浮遊系の細菌と異なる生理学的特徴を持つ。細菌がバイオフィルムを形成することにより様々な問題を引き起こしている。例えば、飲料水配水管内のスライム形成、下水管の腐食、水処理用分離膜のファウリング、冷却水用配管の熱伝導効率の低下などである。さらに、医療分野においては、細菌感染症の多くは病原性微生物が体内に形成するバイオフィルムによって引き起こされる。一度、バイオフィルムが形成されると、抗生物質などの薬剤に対して高い耐性 (浮遊細菌の耐性の 100-1000 倍) を持つ。このように、バイオフィルム形成は水環境分野および医療分野などにおいて極めて重要であるが、これまでの研究は主に、浮遊細菌を対象としたものであり、バイオフィルム形成に係わる遺伝子発現や薬剤耐性メカニズムの獲得については未解明な部分が多く残されている。細菌は、固体表面に付着し、その後小さなコロニーを形成し、構造的にも複雑で成熟したバイオフィルムへと成長する。このバイオフィルム形成には、多くの遺伝子が複雑に関与していると考えられている。さらに、接合による遺伝子の水平伝播も関与している可能性が示唆されているが、詳細は明らかとなっていない。このような背景の基、本研究は、接合プラスミド (F プラスミド) を保持する Escherichia coli をモデル細菌として用い、バイオフィルムの発達および成熟にどのように F プラスミドが関与しているかを検討するために、バイオフィルム形成過程における、E. coli の遺伝子発現プロファイルを DNA マイクロアレイ技術を用いて網羅的に解析している。また、様々な抗生物質に対する耐性をどのように獲得するのか、そのメカニズムについてもこれらの遺伝子発現解析結果と変異株を用いた実験より検討している。さらに、E. coli を用いてバイオフィルム形成のために必要最小限のゲノムはどれだけであるかを実験的に検証するために、膨大な数 (156 株) の遺伝子削除株を用いてバイオフィルム形成能を測定した。得られた最小ゲノムの制御機構や代謝経路についても詳細な考察を加えている。

本論文の各章の内容は以下のようになっている。

第 1 章では、バイオフィルムによって引き起こされる問題や水環境中のバイオフィルムを対象とした研究例、従来の浮遊系を用いた研究の限界についてまとめた上で、これまでのモデル細菌を用いたバイオフィルム形成に関する研究をまとめている。さらに、E. coli のバイオフィルム形成に関

与する重要な遺伝子について言及し、本論文の目的と構成について述べている。
第2章では、E. coli のバイオフィルム形成における F プラスミドの重要性について検討している。接合性 F プラスミドを有する E. coli は、三次元的な複雑な構造をもつ成熟したバイオフィルムを形成するが、F プラスミドを有していない E. coli は、小さなマイクロコロニー様のバイオフィルムしか形成しない。バイオフィルム形成のメカニズムは、F+(F プラスミドを有する細菌) が性線毛により細菌同士を接合し、その後、colanic acids や curli を生産し、これらを接着剤として三次元的なバイオフィルムを形成する。ここで重要な点は、colanic acids や curli の生産は、細菌間の性線毛の接合が引き金となって生じることである。次に、curli の生成にどのような遺伝子が関与しているかを検討するために、バイオフィルム形成過程において DNA マイクロアレイにより網羅的な遺伝子発現解析を行った。その結果、F 因子は、EnvZ/OmpR、RpoS、NH-S などの制御因子を活性化させることにより、curli 生産の間接的な活性因子として働いていることを明らかにしている。
第3章では、E. coli がバイオフィルムを形成するために必要最小限の遺伝子セットとはどれだけであるのかを検討している。この目的を達成するために、膨大な数の遺伝子削除 E. coli 株 (156 株) のバイオフィルム形成能試験、DNA マイクロアレイによる遺伝子発現解析、およびコンピューターによる全ゲノムスケールの代謝経路解析を行っている。実験の結果、E. coli の持つ全ゲノムの 17.6
第4章では、バイオフィルムを形成して存在する E. coli が、どのように抗生物質に対する耐性を獲得するかについて検討している。プラスミド pBR322 を保有する E. coli は、テトラサイクリンとアンピシリンが共存する条件下 (ともに阻害を及ぼしはじめる濃度) で、バイオフィルム形成能が増加することを発見した。この抗生物質耐性獲得およびバイオフィルム形成能向上のメカニズムについて詳細に検討している。検討の結果、テトラサイクリンとアンピシリン共存下では、細胞内に保持するプラスミドのコピー数が増加し、染色体上に存在する薬剤排除ポンプ (EmrY/K、EvgA/S) が活性化される。加えて、テトラサイクリン添加によりテトラサイクリン排除ポンプ (TetA(C)) は活性化され、これに伴って、colanic acids の生成が促進され、バイオフィルム形成能が増加することを明らかとした。
第5章では、本研究で得られた結論を総括し、今後の研究課題についてまとめている。
これらの研究成果は、遺伝子発現レベルにおける、大腸菌 Escherichia coli の複雑なバイオフィルム形成メカニズムおよびバイオフィルムが有する薬剤耐性獲得メカニズムの解明につながる重要な知見であり、環境微生物工学および水環境工学の発展に貢献するところ大なるものがある。よって著者は、北海道大学博士 (工学) の学位を授与される資格あるものと認める。