別紙４（課程博士）

Date of Submission:
平成　28年　7月　15日

| Department<br>環境・生命工学専攻専攻 | Student ID Number<br>学籍番号 | 第 093829 号 |
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| Applicant's name<br>氏名 | 長尾　信義 | |

| Supervisors<br>指導教員 | 梅影　創<br>平石　明 |
|---|---|

**Abstract**

# 論文内容の要旨　(博士)

| Title of Thesis<br>博士学位論文名 | *Rhodovulum sulfidophilum* における細胞外核酸放出機構のゲノム規模による解析とRNA 生産への応用<br>(Genome-wide analysis of the extracellular nucleic acids excretion mechanism in *Rhodovulum sulfidophilum* and its application to RNA production) |
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(Approx. 800 words)

(要旨　1,200 字程度)

海洋性光合成細菌*Rhodovulum sulfidophilum*は、増殖と同時に自身の核酸を細胞外へ放出するという特徴を持つ。また、培養液中の核酸分解酵素活性が著しく低いため、放出されたRNAは培養液中で安定に蓄積する。先行研究によって、本細菌を利用した機能性RNAの細胞外生産技術が開発されている。しかしながら本細菌の核酸放出メカニズムはいまだ明らかになっていない。そこで本研究では、本細菌のゲノム規模による解析を行い、核酸放出メカニズムに関する知見を得ることを目指した。

第一に、本細菌のゲノムDNA配列の決定と全遺伝子の発現解析を行った。ここでは、基準株であるDSM 1374$^{T}$株およびDSM 2351株を解析対象とし、それぞれの株のゲノムDNA配列および遺伝子発現を比較し、既知の表現型の違いと照らし合わせることで、本細菌が持つ遺伝子の機能について考察した。その結果、細胞凝集体（フロック）の形成やアルコール代謝活性、そして形質転換能力の有無など、それぞれの株に特異的な表現型について遺伝子レベルでの知見を得ることができた。

第二に、DSM 1374$^{T}$株が生産するgene transfer agent (GTA)の解析を行った。GTAはある種の細菌が生産するウイルス様粒子であり、内部にはホスト細菌のゲノムDNAがランダムに内包されている。GTAは、細菌群の一部が溶菌することで細胞外に放出されることが知られている。ゲノム解析により、DSM 1374$^{T}$株がGTA構造遺伝子を有していることが明らかとなった。電子顕微鏡観察によって、DSM 1374$^{T}$株はGTA粒子を生産し細胞外に放出していることが示された。また、GTA様粒子に含まれるDNAの長さは約4.5 kbであり、ゲノムDNAがランダムに断片化されたものであることが示された。この結果から、DSM 1374$^{T}$株の細胞外核酸放出にはGTAの生産が関与していることが示唆された。

第三に、細胞外への核酸放出能力が低下した変異体SNK001株を作製し、ゲノムDNA配列解析と遺伝子発現解析を行い、野生株との比較解析を行った。その結果、SNK001株ではゲノムの一部領域において欠落が生じていること、そして欠落領域に含まれる遺伝子群が細胞外核酸放出に関与している可能性が示された。加えて、細胞外RNAの配列を次世代シークエンサーによって網羅的に解析し、細胞外に特異的に放出されているRNAを探索した。

最後に、DSM 1374$^{T}$株の核酸放出機構を応用し、short hairpin RNA (shRNA)の細胞外生産を試みた。その結果、DSM 1374$^{T}$株はshRNA生産に利用可能であることが明らかとなった。この結果は、本細菌を用いたRNA生産手法は様々な機能性RNAに対して応用可能であることを示している。

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A marine phototrophic bacterium *Rhodovulum sulfidophilum* releases DNA and RNA into extracellular milieu during growth. Extracellular nucleic acids are accumulated in culture medium due to low activity of extracellular nucleases. Previously, we succeeded in producing a streptavidin RNA aptamer and hammerhead ribozymes extracellularly by using this bacterium. However, the mechanisms of extracellular nucleic acids excretion remain to be elucidated. Extracellular nucleic acids excretions have been found in many bacterial species, and its mechanism and physiological significance are divergent. Today, genome-wide analysis is powerful tool for comprehensive understanding of organism's property. Unfortunately, genetic information of *Rhodovulum sulfidophilum* were insufficient for genome-wide analysis. In this study, I conducted whole-genome sequencing and RNA-seq analysis of *Rhodovulum sulfidophilum* to obtain insights into the mechanism of extracellular nucleic acids excretion. In addition, I applied this mechanism to functional RNA production method.

First, complete genome sequences and transcriptome analyses were performed on the type strain DSM $1374^T$ and closely related strain DSM 2351. These strains differ in many phenotypes including cell aggregation (flocculation), alcohol metabolism, potential of transformation and extracellular nucleic acids excretion. Genomic and transcriptomic comparative analyses were expected to reveal genotype of the strains and strain-specific genes that may related typical phenotypes. Chromosome of DSM $1374^T$ and DSM 2351 were circular with a size of 4,132,586 bp and 4,454,432 bp, respectively. Number of coding sequences are 3,876 for DSM $1374^T$ and 4155 for DSM 2351. Although two plasmids were conserved in these strains, a unique plasmid was possessed in DSM 2351. The unique plasmid is 60,897 bp and carrying a number of exopolysaccharide (EPS)-related genes. It is well known that bacterial EPS are involved in many biological process, *e.g.* cell aggregation, phage absorption, virulence and protection against antibiotics. Non-conserved regions between two genomes seemed to be acquired by horizontal gene transfer or phage infection. DSM 2351 harbored alcohol dehydrogenase, aldehyde dehydrogenase and restriction enzymes coding genes in non-conserved regions. Both genomes contain prophage regions and structural genes of gene transfer agent (GTA).

Second, GTA of DSM $1374^T$ was subjected to study. GTAs are shaped like bacteriophage particles that containing fragments of host genome. It is known that lysis of subpopulation of bacteria is required to release GTA particle. Previously, we noticed that the extracellular soluble DNA preparation from DSM $1374^T$ contained approximately 4.5 kbp-long DNA,

which is similar in that related bacterium *Rhodobacter capsulatus* produces GTA containing 4.5 kbp DNA. As described above, orthologue of GTA structural genes were found in DSM $1374^T$ genome. Electron microscopy revealed that DSM $1374^T$ produces GTA-like particle into culture medium. This GTA-like particle contained 4.5 kbp DNA that was random fragment from genomic DNA. The 4.5-kbp DNA was not produced when the quorum sensing inhibitor α-cyclodextrin was added into the culture. This result suggested that GTA-like particle production are regulated by quorum sensing. These result suggested that releasing GTA-like particle is associated extracellular nucleic acids excretion in the bacterium.

Third, extracellular nucleic acids excretion deficient mutant SNK001 was obtained from DSM $1374^T$ by long-term subculturing. Amount of extracellular DNA of SNK001 was decreased 30-fold as compared with DSM $1374^T$. Genome sequencing and transcriptome analysis were performed in the strain. In SNK001, three chromosomal regions with size of 33 kbp (region 1), 16 kbp (region 2) and 8.6 kbp (region 3), and Plasmid 2 were deleted. Transcriptome analysis revealed that expression profile of SNK001 was massively altered compared with DSM $1374^T$. Both increased and decreased expression of genes were detected, suggested that transcriptional regulator(s) were defected in SNK001. To determine the genes related to extracellular nucleic acids excretion, extracellular RNA from culture supernatant of DSM $1374^T$ was analyzed by high-throughput sequencing followed by compared with intracellular RNAs. Although most RNAs were detected in intracellular and extracellular samples equally, some RNAs were significantly abundant in extracellular samples. The extracellular-specific RNAs were transcribed from chromosome regions containing deleted region 3 of SNK001. This result suggested that extracellular nucleic acids excretion is occurred by expression of specific genes located in or downstream of the deleted region 3.

Finally, I developed extracellular production method of short hairpin RNAs (shRNAs) using DSM $1374^T$ and engineered plasmid. The shRNA contains a long stem and loop structure which is usually thought to cause transcriptional termination or pause for bacterial RNA polymerases. As a result, shRNAs are successfully produced extracellularly by this system and the yield of the shRNA is almost the same as that of the streptavidin RNA aptamer.

Altogether, this study provides genome sequence, expression profiles and insight of extracellular nucleic acids excretion mechanisms of *Rhodovulum sulfidophilum*. These achievements are excepted to improve functional RNA production method using the bacterium.