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Analysis of functional properties of PirA and ScdA in *Myxococcus xanthus*.

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Abstract

The twin-arginine translocation（Tat）system contributes to export of various important proteins for development and growth for Myxococcus xanthus. In this study, two genes encoding putative Tat protein substrates（a Pirin（PirA）and a short chain dehydrogenase（ScdA））were disrupted by insertion of the kanamycine resistance gene, and the biological function of these proteins were determined. Both mutants showed normal growth in non-osmotic and osmotic condition. When allowed to develop on starvation medium, *pirA* and *scdA* mutants formed normal fruiting bodies, however, the spores of *scdA* mutant were slightly decreased. Under non-osmotic or osmotic-stress, the *pirA* mutant spores germinated at the same rate as wild-type spores.

Key Words: Pirin, Short chain dehydrogenase, Tat system, *Myxococcus xanthus*

Introduction

Myxococcus xanthus is a Gram-negative bacterium that exhibits a complex multicellular developmental cycle [1, 2, 3]. These bacteria live in soil, where they prey on other microbes for food. In response to nutritional stress, hundreds of thousands of the vegetative cells aggregate to form multicellular fruiting bodies containing differentiated myxospores. Once conditions become favorable for growth, the desiccation- and heat-resistant spores can germinate and initiate vegetative growth. *M. xanthus* cells coordinate their multicellular behavior through cell-cell communication by transmitting intercellular signals.

Gram-negative bacteria export a large number of proteins to the periplasmic space. Most proteins destined for export are synthesized with N-terminal extensions, termed signal peptides, and the majority of secretory proteins are exported in an unfolded conformation by the general secretory（Sec）pathway [4]. The Sec pathway is promoted by ATP hydrolysis.

It has recently become clear that most bacteria possess a Sec-independent pathway that mediates the export of proteins in a folded conformation [5, 6, 7]. This alternative pathway has been designated the twin-arginine translocation（Tat）system, because Tat substrates possess a twin-arginine motif（S/T-R-R-x-F-L-K）in signal peptides [8]. In many bacteria, three major components, TatA, TatB and TatC, are the functionally essential constituents of the Tat system. TatA and TatB comprise a single transmembrane N-terminal α-helix followed by a carboxy-terminal domain in the cytoplasm, and TatC usually has six predicted membrane-spanning domains. It is thought that the TatB/TatC complex binds twin-arginine signal peptides of Tat substrates, and then TatA is recruited to the complex to 'coat' the precursor and form a transport channel. The Tat system is energized by the transmembrane proton electrochemical gradient.

We previously reported that a *M. xanthus tatB-tatC* deletion mutant could aggregate and form mounds, but was unable to form fruiting bodies under nutritionally limiting conditions [9]. When *tatB-tatC* mutant vegetative cells were cultured with 0.5 M glycerol, the cell morphology changed to spore-like spherical, but the spores were not resistant to heat and sonication treatments. In contrast to the wild-type strain, the *tatB-tatC* mutant also showed a decreased cell growth rate and a lower maximum cell concentration. These results suggest possibility that the Tat system may contribute to export of various important proteins for development and growth for *M. xanthus*.

By using a Tat substrate recognition program（TATFIND）, *M. xanthus* was predicted to have about 70 putative Tat substrates [9]. In this report, two genes encoding putative Tat substrates（Pirin and short chain dehydrogenase）are disrupted by insertion of kanamycine resistance gene, and the phenotypes of these mutants are discussed.

Material and methods

Bacterial strains and development. The type strain of *M.*

xanthus FB（IFO 13542）was used as the wild-type [10]. *M. xanthus* wild-type and mutant strains were grown in Casitone-yeast extract（CYE）medium, and kanamycin（70 μg ml^{-1}）was added when necessary. For development, *M. xanthus* wild-type and mutant cells were harvested by centrifugation, washed in TM buffer（10 mM Tris-HCl, pH 7.5, and 8 mM $MgSO_4$）and spotted on the surface of CF agar [11]. The plates were incubated at 30°C for 7 days.

Construction of *pirA* and *scdA* disruption mutants by gene replacement. To investigate the biological function of PirA and ScdA, we constructed *pirA* and *scdA* deletion-insertion mutants. First, 2.8- and 2.2-kb fragments containing the *pirA* and *scdA* genes, respectively, were amplified by PCR using as primers, 5' -ACTACTTCTATTCGTCGCCTGAGATGC -3' and 5' -CCGACATCGAGAGGTCAGGGAAC-3' , and 5' -TGACGTTCAGCCCCGCGCTTGCCTCCG-3' and 5' -CCGTTCGTCAACCGCCCGCACCTCCAG-3' , respectively. The PCR products from *pirA* and *scdA* were ligated into pT7Blue-T（Novagen）vector, and these plasmids were designated pPirA and pScdA, respectively. A 1.2-kb DNA fragment containing a kanamycin-resistance（Km^r）gene was amplified by PCR using TnV [12] as a template and a pair of primers. The resulting DNA fragment was inserted into the *Stu*I and *Ehe*I sites of pPirA or *Stu*I sites of pScdA. The disrupted gene constructed as described above was amplified by PCR using the above oligonucleotides. The PCR products thus obtained were introduced into *M. xanthus* cells by electroporation [13].

Growth and stress conditions for *M. xanthus* cells. *M.* xanthus wild-type and mutant strains were precultured in CYE medium at 30°C, and then aliquots of 3 x 10^7 cells were inoculated into 3 ml of CYE medium. Cells were cultured at 30 and 36 °C with shaking at 180 rpm for thermal stress experiments. For osmotic stress experiments, cells were inoculated to 1 x 10^7 cells ml^{-1}, and grown at 30°C in CYE medium containing various concentrations of NaCl or sucrose with shaking. The growth of cells was monitored by measuring optical density at 600 nm, and cell density was determined with a hemocytometer.

Spore germination. Spores were harvested from 6- to 8-day-old fruiting bodies on CF plates, sonicated for 2 min, and treated with heat（60°C for 15 min）. The spores were inoculated to 1 x 10^7 cells ml^{-1} in CYE medium containing up to 0.2 M NaCl or sucrose and incubated at 30°C with continuous shaking until almost all spores in the medium were germinated, and then the number of ungerminated spores in each culture was counted with a hemacytometer.

Results and discussion

Analysis of *pirA*, and *scdA* genes.

By using a Tat substrate recognition program（TATFIND）, we predicted that *M. xanthus* has about 70 putative Tat substrates. We selected two putative Tat substrates, a Pirin（PirA（MXAN4840））and a short chain dehydrogenase（ScdA（MXAN6211））, from these putative Tat substrates. Pirin is a recently identified eukaryotic protein implicated in transcriptional activation and apoptosis [14]. Homologues of Pirin are highly conserved in both prokaryotes and eukaryotes, but their function remains poorly understood. ScdA is homologous to short chain dehydrogenases that also has homology to *M. xanthus* contact-dependent extracellular C-signal（Fig. 1）. The C-signal acts as morphogen and induces distinct responses at discrete thresholds during the developmental process that leads to formation of multicellular, spore-filled fruiting bodies [15]. The mature C-signal protein is synthesized by N-terminal proteolytic processing of the full-length CsgA protein by a serine protease [16]. ScdA also has a similar amino acid sequences, which the cleavage site is located between amino acid residues 60 and 68 in CsgA. Therefore, we investigated whether ScdA also acts as a C-signal. These encoding genes were searched from the *M. xanthus* genomic database of the Institute for Genomic Research（TIGR）.

pirA encoded 318 amino acid residues deduced from its nucleotide sequence with a molecular mass of 34.9 kDa. A computer search using the BLAST program revealed that the PirA shares significant sequence homology to the Pirin. PirA

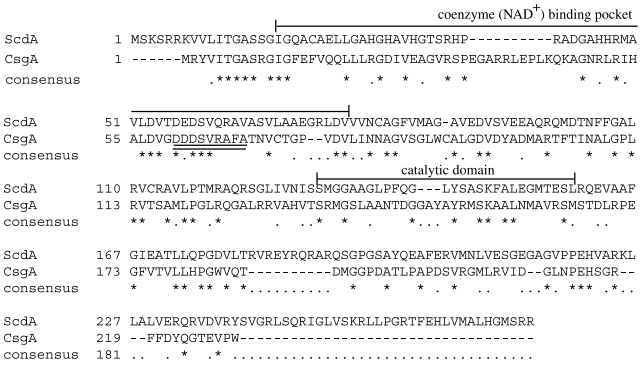

Fig. 1. Alignment of the deduced ScdA with the C-signal protein（CsgA）of *M. xanthus*. The double line shows a location of cleavage site region of CsgA. Identical residues are marked by an asterisk.

was 49, 50 and 48% identical to the Pirin of *Xanthomonas axonopodis*, the Pirin of *Chromobacterium violaceum*, and the Pirin of *Thiomicrospira crunogena*, respectively.

scdA encoded 274 amino acid residues deduced from its nucleotide sequence with a molecular mass of 29.5 kDa. A computer search using the BLAST program revealed that the ScdA showed 41, 41, and 28% identity to the short chain dehydrogenase of *Bacteroides thetaiotaomicron,* the short chain dehydrogenase of *Flavobacterium johnsoniae*, and CsgA of *M. xanthus*, respectively.

Construction of *pirA* and *scdA* mutants.

To investigate the biological function of PirA and ScdA, we constructed *pirA* and *scdA* single mutants. *pirA*::Km^r and *scdA*::Km^r were constructed by inserting a kanamycin resistance gene cassette from TnV into the the *Stu*I and *Ehe*I sites of *pirA*, and the *Stu*I sites of *scdA*, respectively (Fig. 2). Using PCR and restriction enzyme analyses, we confirmed that the kanamycin-resistance gene was inserted into the *pirA* or *scdA* gene, respectively, on chromosome of *M. xanthus* mutants.

Phenotypes of *pirA* and *scdA* mutants.

(i) **Cell growth.** The *pirA* and *scdA* mutants showed normal growth in CYE medium at optimal (30°C) temperature (data not shown). When cultured at high temperature (37°C), no significant difference was observed in the growth at high temperature between the wild type, *pirA* and *scdA* mutants.

(ii) **Osmosensitivity.** Hihara *et al*. reported that a cyanobacterial gene encoding an ortholog of Pirin is induced under stress conditions [17]. The *pirA* mutant grew as well as the wild type did under osmotic stress (0.2 M NaCl or 0.2 M sucrose, data not shown), suggesting that PirA is not involved in the adaptation to osmotic stress during vegetative growth.

(iii) **Development.** To study the functions of PirA and ScdA in development, the wild-type and these mutant strains were cultured on starvation medium (CF agar). The wild-type strain and all mutant strains formed normal fruiting bodies after 2 to 3 days of incubation on CF plates with 1.5% agar. Within the fruiting bodies of the wild type, *pirA* and *scdA* mutants, vegetative cells were converted into spherical myxospores. However, the spore yield of the *scdA*-mutant strain was approximately 70% of that of the wild-type strain (Fig. 3). There was no obvious difference in spore yields between wild type and *pirA* mutants (data not shown).

(iv) **Glycerol-induced spores.** *M. xanthus* vegetatively growing cells can form spores when exposed to high levels of glycerol [18]. The glycerol-induced spores are more resistant to ultraviolet irradiation, sonic vibration, and heat than are vegetative cells [19]. By 1.5 to 2 h after the introduction of the glycerol, the rod-shaped vegetable cells of wild type and mutants had completed the morphological alteration to spheres. There was no significant difference in the final numbers of spherical cells between wild-type and mutant cells.

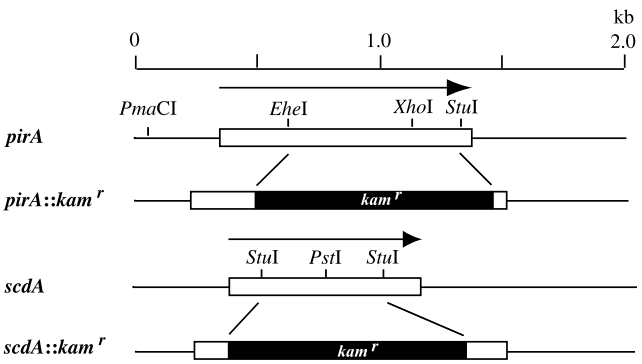

Fig. 2. Restriction map of the *pirA* and *scdA* genes of *M. xanthus*. Lines with arrows indicate orientation. The 0.66-kb *Ehe*I-*Stu*I fragment of *pirA* or the 0.54-kb *Stu*I fragment of *scdA* was replaced by the Km^r gene, respectively.

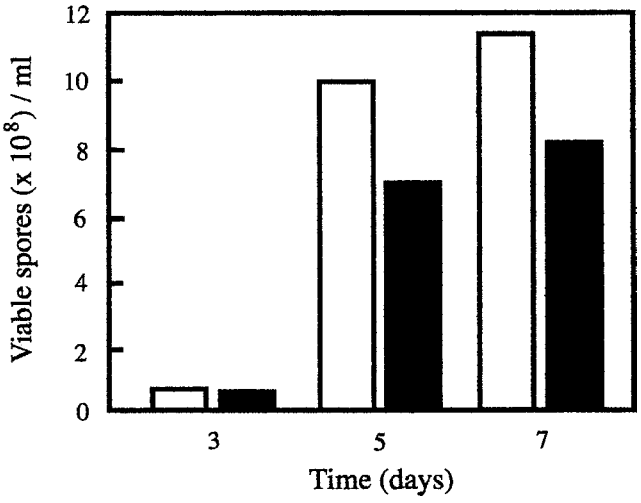

Fig. 3. Spore formation of *M. xanthus* wild-type, and *scdA* mutant strains. The cells were developed on CF agar, and numbers of spores from wild-type (open box) and *scdA* mutant (closed box) strains were counted using a Petroff-Hausser counter. Data are the means of duplicate experiments.

(v) **Spore germination.** Under non-stress conditions, spores of all mutants germinated normally in CYE medium (Fig. 4). The mutant spores began to germinate at about 24 h of incubation, and elongated into rod-shaped cells. Under osmotic-stress, the germination of wild-type and mutant spores was delayed, and the wild-type and mutant spores germinated in about the same time.

Pirin is an identified protein in eukaryotes as a transcription cofactor or as an apoptosis-related protein [20, 21, 22]. Although Pirin is highly conserved from bacteria to human, there have been no reports on the function of prokaryotic Pirin. We investigated the function of PirA using *pirA* mutant, however, the difference of phenotype between wild-type and *pirA* mutant were not observed. From *M. xanthus* genomic data, *M. xanthus* is predicted to have five Pirin-like proteins. The *pirA* mutant may be complemented by these Pirin homologs.

On the other hand, the formation of spores in *scdA* mutant under starvation condition was slightly decreased when compared with that of wild type. However, we could not judge whether the ScdA functions as a C-signal, because the difference in spore formation between wild type and *scdA* mutant was slightly.

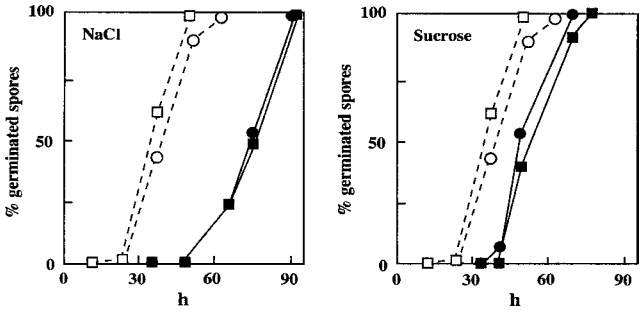

Fig. 4. Spore germination of *M. xanthus* wild-type (circles) and *pirA* mutant (squares), and spores in CYE medium containing 0.2 M NaCl or sucrose (closed symbols) or without either (open symbols). The number of ungerminated spores in each culture was counted with a hemacytometer.

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Myxococcus xanthus におけるPirAとScdAタンパク質の機能解析

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我々はtwin-arginine タンパク質輸送（Tat）システムが*M. xanthus*において生育や分化における重要なタンパク質をペリプラズマ画分に輸送していることを報告している．この研究ではTatシステムによって輸送されていると推定されたPirin（PirA）とShort-chain dehydrogenase（ScdA）タンパク質をコードしている遺伝子を破壊した変異株を用いてそれらのタンパク質の機能解析を行った．それらの変異株は非浸透圧あるいは浸透圧条件下でも正常な生育がみられ，また，飢餓による分化の誘導により，両変異株は正常な子実体を形成したが，*scdA*変異株において胞子数は野生株のそれより減少した．*pirA*変異株における胞子の発芽は非浸透圧あるいは浸透圧条件下において野生株と変化はみられなかった．