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Short Communication

A *sbiT-sbiRS-gloIo* regulatory circuit is involved in oxidative stress tolerance of *Stenotrophomonas maltophilia*

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Abstract The *sbiT-sbiR-sbiS* operon of *Stenotrophomonas maltophilia* encodes an inner-membrane protein SbiT and a SbiS-SbiR two-component regulatory system. A *sbiT* mutant displayed a growth defect in LB agar. Mechanism studies revealed that *sbiT* deletion resulted in SbiSR activation and *gloIo* upregulation, which increased intracellular ROS level and caused growth defect.

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Introduction

Oxidative stress is unavoidable in aerobic microorganisms. Reactive oxygen species (ROS) are produced through bacterial aerobic respiration and generated by the host immune system. To protect themselves against ROS-induced oxidative stress, bacteria have evolved different ROS defense mechanisms, including enzymatic ROS scavengers, non-enzymatic molecules, and efflux pumps.[1]

The glyoxalase system comprises a set of enzymes that detoxify reactive electrophilic species (RES). Glyoxal (GO) and methylglyoxal (MGO), the members of RES, can non-enzymatically modify nucleic acids and proteins, causing mutations. Glyoxalases include glutathione (GSH)-dependent enzymes, glyoxalase I (GloI) and glyoxalase II (GloII), and GSH-independent glyoxalase III (GloIII).[2] Although many bacterial *gloI* genes have been annotated in bacterial genomes, few bacterial GloI have been characterized.

Two-component regulatory systems (TCSs), composed of a sensor kinase (SK) and a response regulator (RR), are important mediators of signal transduction.[3] Genes encoding SK and RR are generally organized in an operon. However, it is sometimes observed that additional genes are in the same operon as the SK and RR genes. The proteins encoded by these additional genes may modulate the activity of SK or RR at the post-translational level.

Stenotrophomonas maltophilia is an emerging opportunistic pathogen in immunocompetent individuals. In our recent study, we disclosed a *sbiT-sbiR-sbiS* operon, a member of ferric uptake regulator (Fur) regulon, and elucidated its role in iron homeostasis.[4] The proteins encoded by *sbiR* and *sbiS* comprise a TCS, whose activation is transcriptionally regulated by Fur and post-translationally modulated by SbiT via SbiT—SbiS interaction. Activated SbiR upregulates the expression of SmeDEF and SbiAB pumps, which contribute to stenobactin secretion.[4] In that study, we noticed that a *sbiT* mutant, KJΔSbiT, displayed a compromised growth in LB agar.[4] In this study, we attempted to elucidate the underlying mechanism for the *ΔsbiT* phenotype.

Materials and methods

Transcriptome analysis

Total RNA isolation, DNase treatment, rRNA depletion, adapter-ligated cDNA library construction and enrichment, and cDNA sequencing, were performed as described previously.[4] The output R1 reads were mapped to the genome of K279a. Gene enrichment analysis was conducted using topGo v2.44.0 with Fisher's exact test and Weight 01 algorithm. The total number of reads per gene was normalized by transcripts per kilobase million (TPM) values.

Construction of deletion mutant KJΔGloIo

Two DNA fragments flanking N-terminus and C-terminus of *gloIo* gene were amplified via PCR using the primer sets of GloIoN-F/R and GloIcC-F/R (Suppl. Table 1), respectively, and subsequently cloned into pEX18Tc, generating pΔGloIo (Suppl. Table 1). Transconjugant selection and double crossover mutant confirmation were performed as described previously.[4]

Intracellular iron level determination

The logarithmical phase cells were collected and resuspended in 2 mL of Milli-Q water. The cell suspension was sonicated, centrifuged, and filtered through a 0.45-mm-pore-size Millipore. Bacterial aliquot was collected for inductively coupled plasma-mass spectrometry (ICP-MS) assay (Agilent 7700e instrument, Agilent Technologies, USA). Cell numbers in bacterial aliquot were determined by CFU counting. Iron level was normalized by the cell numbers. The commercially available iron standards were included as a control.

Intracellular ROS level determination (DCFH-DA assay)

The logarithmical-phase bacterial culture was treated with DCFH-DA (final concentration 0.01 μM) for 1 h at 37 °C. A cell-free LB broth with DCFH-DA was used as a blank control. To monitor DCFH-DA oxidation, the fluorescence intensity was measured at 488 nm excitation and 520 nm emission. The bacterial mass was determined by recording the optical density at 450 nm (OD_{450nm}). Intracellular ROS level was expressed as fluorescence intensity/OD_{450nm}.

Glyoxalase I activity assay

The glyoxalase I activity was determined using Glyoxalase I activity Assay Kit (Sigma—Aldrich, Missouri, USA) and activity was calculated according to the formula provided by the manufacturer. The specific activity of glyoxalase I was expressed as glyoxalase I activity/mg protein. Protein concentration was determined using the Bio-Rad protein assay reagent, with bovine serum albumin as a standard.

Results

Inactivation of *sbiT* results in a growth defect in a *sbiRS*-dependent manner

KJΔSbiT exhibited a growth defect in LB agar (Suppl. Fig. 1A), and this phenotype was confirmed by monitoring bacterial growth in LB broth (Suppl. Fig. 1B). Furthermore, we noticed that growth defect observed in KJΔSbiT was restored to the wild-type level when *ΔsbiRS* was introduced into the KJΔSbiT (Suppl Fig. 1). Thus, inactivation of *sbiT* results in a growth defect in a *sbiSR*-dependent manner.

KJΔSbiT experiences a ROS stress, not an iron-overload stress

For the *ΔsbiT*-mediated growth defect, we assumed that *sbiT* deletion leads to SbiSR TCS activation, which makes KJΔSbiT experience an iron-overload stress. To test this hypothesis, 2,2′-dipyridyl (DIP) tolerance and intracellular iron levels were investigated. KJ and KJΔSbiT displayed comparable viability in the DIP-containing plates (Fig. 1A) and comparable intracellular iron levels (Fig. 1B).

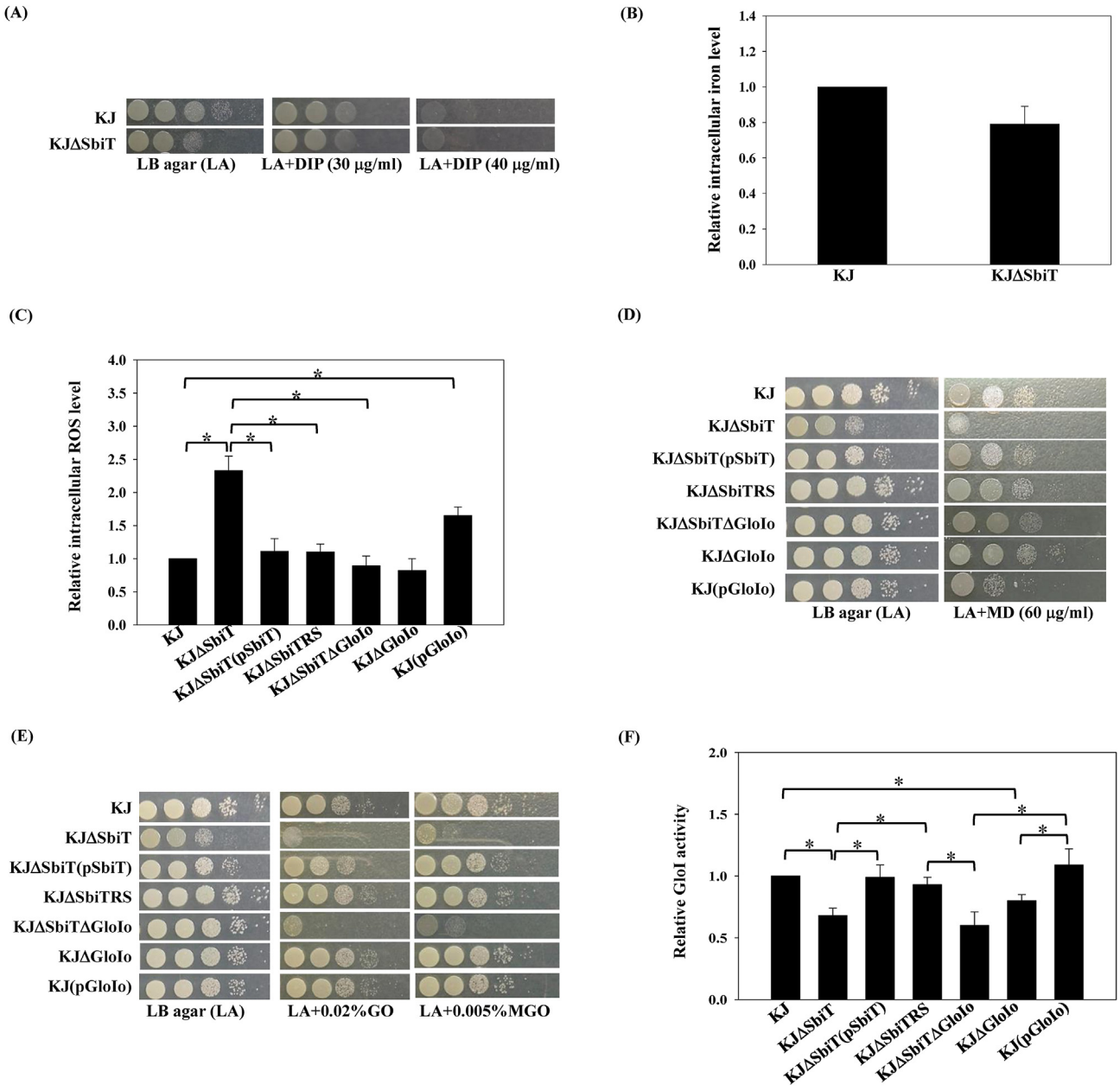

Figure 1. Role of *sbiTRS* operon and *gloIo* in iron-depleted, ROS, and RES stress.
(A) The impact of *sbiT* inactivation on DIP tolerance. Logarithmic phase bacterial cells of 2×10^5 cfu/μl were serially 10-fold diluted. Bacterial suspensions (5 μl) were spotted onto LB agar with and without DIP. After 24-h incubation at 37 °C, the growth of bacterial cells was observed. DIP, 2, 2-dipyridyl. (B) The intracellular iron levels of wild-type KJ and KJΔSbiT. The amount of iron in the logarithmically grown wild-type KJ and KJΔSbiT was determined by inductively coupled plasma mass spectrometry (ICP-MS). The relative iron level was calculated using the iron level of KJ cells as 1. Bars represent the average values from three independent experiments. Error bars represent the standard errors of means. (C) The intracellular ROS levels of KJ and its derived constructs. The bacterial cells tested were cultured in LB medium containing DCFH-DA for 5 h and the fluorescence at 550 nm was determined. The relative fluorescence is normalized to the fluorescence of wild-type KJ. *$P < 0.01$, significance calculated by Student's *t* test. (D) The MD susceptibility of KJ and its derived constructs. Logarithmic phase bacterial cells of 2×10^5 cfu/μl were serially 10-fold diluted. Bacterial suspensions (5 μl) were spotted onto LB agar with and without MD. After 24-h incubation at 37 °C, the growth of bacterial cells was observed. MD, menadione. (E) Cell viabilities of wild-type KJ and its derived constructs in GO- and MGO-containing medium. Logarithmic phase bacterial cells of 2×10^5 cfu/μl were serially 10-fold diluted. Bacterial suspensions (5 μl) were spotted onto LB agar with and without GO (or MGO). After 24-h incubation at 37 °C, the growth of bacterial cells was observed. (F) The intracellular glyoxalase I activities of wild-type KJ and its derived constructs. The bacterial cells tested were cultured in LB medium containing DCFH-DA for 5 h and the fluorescence at 550 nm was determined. The relative GloI activity was calculated using the GloI activity of wild-type KJ cells as 1. Bars represent the average values from three independent experiments. Error bars represent the standard errors of means. *$P < 0.01$, significance calculated by Student's *t* test.

Given that ROS levels and iron toxicity are closely associated in vivo, we wondered whether growth defect of KJΔSbiT is related to intracellular ROS levels. Relative to that in wild-type KJ, the ROS level in KJΔSbiT had a 2.33 ± 0.22-fold increment and reversed to wild-type level in KJΔSbiT(pSbiT) and KJΔSbiTRS (Fig. 1C). Furthermore, KJΔSbiT was more susceptible to menadione (MD) than wild-type KJ, but KJΔSbiT(pSbiT), KJΔSbiTRS, and KJ had comparable susceptibility to MD (Fig. 1D). Collectively, KJΔSbiT experiences a ROS stress, not an iron-overload stress.

GloIo upregulation in KJΔSbiT contributes to increased ROS level and MD susceptibility

Based on the above results, we speculated that some gene(s) of the SbiSR regulon are involved in the *ΔsbiT*-mediated decrease in MD tolerance. RNAseq transcriptome analysis of KJ, KJΔSbiT, and KJΔSbiTRS was conducted to reveal the SbiSR regulon. The gene expression changes greater than threefold was defined as significant. We aimed to search for candidate gene(s) whose expression was significantly upregulated (or downregulated) in KJΔSbiT and tended to revert to wild-type KJ levels in KJΔSbiTRS. In total, 68 genes matched this criterion (Suppl. Table 2). Among the 68 candidate genes (Suppl. Table 2), *smlt0186* transcript showed the greatest change in KJΔSbiT. *smlt0186* expressions in KJ, KJΔSbiT, and KJΔSbiTRS were validated by qRT-PCR (Suppl. Fig. 2). *smlt0186* is annotated as a glyoxalase gene in *S. maltophilia* K279a. Genome-wide survey on K279a revealed 12 annotated glyoxalase genes (Suppl. Table 3). Phylogenetic analysis of the 12 Glos and the characterized Glos from other bacteria was conducted (Suppl. Fig. 3). Based on the phylogenetic analysis and the following findings, we designated Smlt0186 as GloIo (O indicates oxidative stress).

To clarify contribution of *gloIo* upregulation to *ΔSbiT*-mediated decrease in MD tolerance, a *sbiT* and *gloIo* double deletion mutant, KJΔSbiTΔGloIo, was constructed. Compared to KJΔSbiT, KJΔSbiTΔGloIo reverted ROS level and MD susceptibility to wild-type level (Fig. 1C & D), indicating that GloIo upregulation contributes to *ΔSbiT*-mediated increase in ROS level MD susceptibility. The impact of *gloIo* deletion or overexpression on the ROS level and MD susceptibility of wild-type KJ were also assessed. Compared with wild-type KJ, KJΔGloIo displayed comparable ROS level and MD susceptibility; however, KJ(pGloIo) had increased ROS level and MD susceptibility (Fig. 1C & D), further supporting the positive contribution of GloIo upregulation to ROS level and MD susceptibility.

GloIo upregulation is irrelevant to *ΔsbiT*-mediated decrease of GO/MGO tolerance and GloI activity.

Given that glyoxalase contributes to RES detoxification,[2] we investigated GO/MGO tolerance and GloI activities of wild-type KJ and its derived constructs. Compared to wild-type KJ, KJΔSbiT displayed decreased GO/MGO tolerance (Fig. 1E) and GloI activity (Fig. 1F) and these compromises were reverted to wild-type levels when *sbiT* was complemented or *sbiRS* was further deleted (Fig. 1E & F). However, *gloIo* deletion from KJΔSbiT did not further decrease GO/MGO tolerance (Fig. 1E) and GloI activity (Fig. 1F), indicating that *gloIo* upregulation is irrelevant to *ΔsbiT*-mediated decreased of GO/MGO tolerance and GloI activity. We wonder whether GloIo harbors GloI activity; thus, KJΔGloIo, a *gloIo* deletion mutant, and KJ(pGloIo), a *gloIo* overexpression strain, were subjected to GloI activity assay. KJΔGloIo displayed lower GloI activity than wild-type KJ and KJ(pGloIo) (Fig. 1F), supporting the presence of GloI activity in GloIo.

Discussion

Glyoxalase I (GloI) is a member of the glyoxalase system and known to be involved in RES detoxification.[2] In this study, we revealed that the GloIo protein of *S. maltophilia* has a significant impact on ROS stress and is less related to RES stress. A bacterial GloI displaying additional functions beyond RES detoxification has been reported in *E. coli*. The GloI of *E. coli* functions in potassium homeostasis because GloI activity is proportional to the activation level of the KefB and KefC potassium efflux pumps.[5] In this study, we firstly report that *gloIo* overexpression resulted in increased intracellular ROS levels (Fig. 1C) and MD susceptibility (Fig. 1D), but hardly impacted on GO/MGO tolerance (Fig. 1E). Therefore, GloIo may represent an unrecognized class of glyoxalase I enzymes, harboring some unidentified enzyme activities, in addition to glyoxalase activity. We speculated that GloIo activity may contribute to the increase in ROS levels and/or decrease in ROS alleviation.

sbiTRS operon, a member of Fur regulon, is moderately expressed in logarithmically grown wild-type KJ, but SbiSR TCS keeps in a resting condition due to SbiT—SbiS interaction.[4] In an iron-depleted condition, Fur loses its binding affinity to Fur box and the SbiT—SbiS interaction disappears, resulting in the high-level activation of SbiSR TCS, which provides a benefit to bacteria for iron source acquisition.[4] In this study, we further demonstrated that KJΔSbiT displayed a growth defect in LB agar, but not in DIP-containing LB agar (Fig. 1A), indicates that activation of SbiSR TCS in an iron replete condition is detrimental to bacteria, making them more sensitive to ROS. Thus, the two-brake implement of Fur and SbiT prevents bacteria from SbiSR over-activation in an iron replete condition.

Increased intracellular ROS level seems to be a shortcoming for bacteria; however, the impact of ROS on the development of antibiotic resistance cannot be ignored. Several antibiotic resistance mechanisms are triggered by ROS[6,7] and some oxidative stress alleviation systems can cross-protect bacteria from antibiotic challenges.[8—10] Therefore, a bacterium with an elevated intracellular ROS level, such as KJΔSbiT in this study, is prone to resistance development.

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CRediT authorship contribution statement

Cheng-Mu Wu: Formal analysis, Methodology, Writing — original draft, Writing — review & editing. **Yi-Tzu Lee:** Conceptualization, Data curation, Resources, Writing — original draft, Writing — review & editing. **Hsu-Feng Lu:** Conceptualization, Data curation, Writing — review & editing. **Yen-Ling Lin:** Data curation, Methodology, Writing — review & editing. **Tsuey-Ching Yang:** Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing — review & editing.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.07.005.