Role of Corynebacterium glutamicum sprA Encoding a Serine Protease in glxR-Mediated Global Gene Regulation

Eun-Ji Hong1, Joon-Song Park1*, Younhee Kim2, Heung-Shick Lee1*

1 Department of Biotechnology and Bioinformatics, Korea University, Sejong-ro, Sejong-si, Korea, 2 Department of Oriental Medicine, Semyung University, Checheon, Chungbuk, Korea

Abstract

The global regulator glxR of Corynebacterium glutamicum is involved in many cellular activities. Considering its role, the GlxR protein likely interacts with other proteins to obtain, maintain, and control its activity. To isolate proteins interacting with GlxR, we used a two-hybrid system with GlxR as the bait. Subsequently, the partner, a subtilisin-like serine protease, was isolated from a C. glutamicum genomic library. Unlike glxR, which showed constitutive expression, the expression of sprA, encoding a serine protease, was maximal in the log phase. Purified His6-SprA protein underwent self-proteolysis and proteolyzed purified GlxR. The proteolytic action of SprA on GlxR was not observed in the presence of cyclic adenosine monophosphate, which modulates GlxR activity. The C. glutamicum sprA deletion mutant (ΔsprA) and sprA-overexpressing (P180-sprA) strains showed reduced growth. The activity of isocitrate dehydrogenase (a tricarboxylic acid cycle enzyme) in these strains decreased to 30–50% of that in the wild-type strain. In the P180-sprA strain, proteins involved in diverse cellular functions such as energy and carbon metabolism (NCgl2809), nitrogen metabolism (NCgl0049), methylation reactions (P180), phosphate uptake [18], and cell resuscitation [19]. Further, Kohl et al [20] analyzed the whole genome using in silico tools in combination with in vitro analysis and predicted that the GlxR regulon comprises many genes (96 genes in 53 transcription units) presumed to be involved in diverse cellular activities such as carbohydrate metabolism, aromatic compound degradation, glutamate uptake and nitrogen assimilation, fatty acid biosynthesis, stress response, and resuscitation. The global function of GlxR has recently been confirmed and expanded by Jungwirth et al [21] and

Introduction

Corynebacterium glutamicum is a non-pathogenic, Gram-positive organism that belongs to the order Actinomycetales. Based on its metabolic capability, this organism has been widely used in the industrial production of nucleotides and amino acids [1]. Therefore, the catabolic and anabolic pathways associated with amino acid metabolism have attracted scientific attention and have been extensively analyzed. Moreover, the availability of genome sequence data [2–4] has significantly increased the understanding of gene expression and regulation on a global scale [5].

The global regulator glxR of C. glutamicum was initially identified through its capability to repress glyoxylate bypass genes [6]. The bypass, mediated by isocitrate lyase (ICL) and malate synthase (MS), is not required by cells grown with glucose as the sole carbon source, and therefore the genes mediating the bypass are repressed by glxR. However, when cells are provided with two-carbon compounds, such as acetate, the bypass plays a critical role because it conserves carbon by bypassing the CO2-generating steps of the tricarboxylic acid cycle. ICL (encoded by aceA) catalyzes the conversion of the tricarboxylic acid cycle intermediate isocitrate into glyoxylate and succinate, and MS (encoded by aceB) catalyzes the subsequent condensation of glyoxylate with acetyl-coenzyme A to produce malate. Furthermore, transcriptional regulators such as RamA and RamB are also involved in the regulation of aceA and aceB expression [7–9].

GlxR is a cyclic adenosine monophosphate (cAMP)-binding transcriptional regulator [10] that belongs to the cAMP receptor protein/luminate and nitrate reduction regulator (CRP/FNR) family of proteins and shows homology to the global regulator CRP from Escherichia coli [6]. In addition to its role in acetate metabolism, glxR is involved in many other cellular activities, such as carbon metabolism [6,11–14], energy metabolism [15], lipid metabolism [16], anaerobic nitrate respiration [17], inorganic phosphate uptake [18], and cell resuscitation [19]. Further, Kohl et al [20] analyzed the whole genome using in silico tools in combination with in vitro analysis and predicted that the GlxR regulon comprises many genes (96 genes in 53 transcription units) presumed to be involved in diverse cellular activities such as carbohydrate metabolism, aromatic compound degradation, glutamate uptake and nitrogen assimilation, fatty acid biosynthesis, stress response, and resuscitation. The global function of GlxR has recently been confirmed and expanded by Jungwirth et al [21] and
To analyze GlxR-bound target DNA sequences isolated from cells via immunoprecipitation. A total of 14% of C. glutamicum genes may be under the direct transcriptional control of GlxR [23].

Despite the importance of GlxR as a global regulator, many questions regarding its mechanism and the control of its action remain largely unanswered, partly owing to the essential role of glxR in cell physiology. For example, in earlier studies, glxR deletion mutants could not be isolated [6,13] or grew too slowly for study when isolated [24]. However, Park et al [25] were able to isolate a glxR deletion mutant; they demonstrated that the mutant strain had a severe growth defect and that glxR repressed glyoxylate bypass genes. Incidentally, GlxR functions as a dual regulator that can be a repressor or an activator depending on its target genes. The position of its consensus binding site TGTGA-[20] relative to the transcriptional start site target genes. The position of its consensus binding site TGTGA-[20] which harbored a glutaric acid (C. glutamicum) as an interacting partner for SprA. Given the physiological and biochemical data, we propose a role for sprA in glxR-mediated global gene expression.

Materials and Methods

Bacterial Strains and Growth Conditions

C. glutamicum AS019E12 [27] was used to construct HL1385 and HL1389 harbored the sprA-complementing plasmid pSL555 and sprA-overexpressing plasmid pSL509, respectively. E. coli DH10B Invitrogen) was used for the construction and propagation of plasmids. E. coli BL21-CodonPlus (DE3)-RIIL (Stratagene) and E. coli DH5α (Bethesda Research Laboratories) were used for the expression of histidine-tagged SprA (His6-SprA) and maltose-binding protein (MBP)-fused GlxR (MBP-GlxR), respectively. E. coli and C. glutamicum strains were cultured in Lauria-Bertani broth at 37°C and MB medium at 30°C, respectively [27]. MCGC minimal medium for C. glutamicum was prepared as described previously [28]. Glucose and acetate were added as carbon sources to the MCGC minimal medium at 1% and 2% (w/v), respectively. Selective and nonselective plates (BacterioMatch II Two-Hybrid System, Agilent Technologies) for E. coli XLI-Blue MRF’ can be prepared as described [29]. Antibiotics were added at the following concentrations (µg mL−1): 50 ampicillin, 5 tetracycline, 20 chloramphenicol, and 25 kanamycin.

DNA and RNA Analyses

Plasmids were introduced into C. glutamicum cells through electroporation [30]. Total RNA isolation for C. glutamicum was performed using the FastPrep24 system (MP Biomedicals). Polymerase chain reaction (PCR) was performed as previously described [6]. cDNA conversion and real-time quantitative PCR (RT-qPCR) were also performed as previously described [29]. CFX96 Real-Time PCR Detection System (Bio-Rad) was used for gene expression analysis. Standard curves, expression normalization, and standard error values were obtained using CFX Manager software ver. 1.5 (Bio-Rad), which employs the ΔΔCt method, and 16S rRNA was used for normalization. RT-qPCR products were verified by melting curve and peak analyses. The following primers were used: glxR, 5’-CAGCAGACAGCTCTGGATGC-3’ and 5’-TTAGCCAGCTGCAAAAG-3’; NCgl0550, 5’-AGCTTGGCCGCTCTGTTG-3’ and 5’-AGTTGCTATGTTGAGCAGTTGGA-3’; NCgl0719, 5’-AGAGCCACGCTGTTGTCAC-TATT-3’ and 5’-TCGAGAGATCATGTTTTGAGCAGCA-3’; NCgl0938, 5’-AGCGGAGTCGTCGCTGAGG-3’ and 5’-TAGTCTCCTGACCGGAACTCCGA-3’; NCgl2989, 5’-TCACCTCTGGGAAAGCTCCGATAGAC-3’ and 5’-ATTAGCCGACCTCTCGACCAAAAG-3’; and 16S ribosomal RNA, 5’- CGGCGGACTAGCTTTGTTGG-3’ and 5’-TGCCGCGGTGTCAGTCAGTT-3’.

Plasmid and Strain Construction

Plasmid pSL500 harboring glxR cloned into the pBT vector (Agilent Technology) was constructed by introducing the EcoRI and BamHI-digested fragment, which was amplified from the C. glutamicum chromosome with primers 5’-CCGGGATTCCTGGTGATATTACAGCTGAAACTGTCAGTACAGCCTGAAAGCCGGAAGAATGCTCAATG-3’ and 5’-CCGGGATTCCTGGTGATTACAGCTGAAACTGTCAGTACAGCCTGAAAGCCGGAAGAATGCTCAATG-3’. The plasmids pSL500, pSL501 (pTRG-NCgl0550), pSL503 (pTRG-NCgl1430), pSL504 (pTRG-NCgl2510), and pSL505 (pTRG-NCgl1535) were constructed using the procedure described above but with the following primers: pTRG-NCgl2548, 5’-CCGGGATTCCTGGTGATATTACAGCTGAAACTGTCAGTACAGCCTGAAAGCCGGAAGAATGCTCAATG-3’ and 5’-CCGGGATTCCTGGTGATATTACAGCTGAAACTGTCAGTACAGCCTGAAAGCCGGAAGAATGCTCAATG-3’. The plasmid pSL501 expressing the His6-SprA fusion protein was constructed via amplification of sprA using the primers 5’-CCGGGATTCCTGGTGATATTACAGCTGAAACTGTCAGTACAGCCTGAAAGCCGGAAGAATGCTCAATG-3’ and 5’-CCGGGATTCCTGGTGATATTACAGCTGAAACTGTCAGTACAGCCTGAAAGCCGGAAGAATGCTCAATG-3’. The plasmids pSL500 and pSL501 were digested with the HindIII and XhoI digest and ligating the 1.3-kb PCR product into the HindIII- and XhoI-digested pET28a vector. The plasmid Psl509 harboring sprA gene was amplified using the primers 5’-CCGGGATTCCTGGTGATATTACAGCTGAAACTGTCAGTACAGCCTGAAAGCCGGAAGAATGCTCAATG-3’ and 5’-CCGGGATTCCTGGTGATATTACAGCTGAAACTGTCAGTACAGCCTGAAAGCCGGAAGAATGCTCAATG-3’.
with the primers sprAforF 5'-TTTGGGCGCCGGTGGATACGAAGC-3' and sprAforR 5'-CCGGGATCCTGGATGATGCATTCTGAAAC-3'. The 310 bp promoter region was amplified with the primers sprFpl 5'-CGCGCTCGAGTACCCGTTGGCGCA-3' and sprApR 5'-TTATCCACGCGCCCAAAATGTCGCCGC-3'. The amplified 1.3-kb and 310-bp fragments were ligated and inserted into the pMT1 vector [27] previously digested with BamHI and XbaI.

The C. glutamicum ΔsprA mutant strain was constructed according to a method described by Schäfer et al [32]. Briefly, a DNA fragment was prepared from the C. glutamicum genome using PCR with the following primers: F1, 5'-CCGGGATCCTGGATGATGCATTCTGAAAC-3', R1, 5'-CCGGGGATCCTGGATGATGCATTCTGAAAC-3'; F2, 5'-ATTGGGACGGCACAATTGCCTGGTCTATCC-3'; R2, 5'-CCGGGATCCTGGATGATGCATTCTGAAAC-3'. The amplified fragment was cloned into the pGEM-T Easy vector (Promega). The BamHI fragment was then isolated and inserted into BamHI-digested pK9mobsaB [32]. Subsequent procedures were conducted as previously described [30,33], and the chromosomal deletion of sprA in C. glutamicum HL1385 was verified by PCR with the primers F1 and R2.

Two-hybrid System

A C. glutamicum genomic library was prepared as previously described [29]. The BacteroMatch II Two-Hybrid system (Agilent Technology) was used according to manufacturer instructions. Briefly, the two plasmids, pBT and pTRG, containing the “bait” and “target” genes, respectively, were used to transform E. coli simultaneously. Protein–protein interactions were screened based on the expression of his3 and adh4, which confer histidine prototrophy (His+) and streptomycin resistance (Str+), respectively. For screening, 50 ng of each pSL500 (i.e., pBT-glxR) and target library DNA were introduced into reporter cells and spread onto selective media (His- and Str+). Colonies were isolated and the plasmids in the growing cells were analyzed.

Protein Purification and Proteolytic Assays

MBP-GlxR was purified as previously described [6]. His6-SprA was overexpressed, isolated, and refolded on a HisTrap FF column (GE Healthcare) as described in the Recombinant Protein Purification Handbook (GE Healthcare). Briefly, we grew E. coli BL21-CodonPlus (DE3)-RIL cells harboring plasmid pSL510 in 300 mL Luria-Bertani broth to an optical density of 0.5 at 600 nm, added isopropylthio-β-galactoside to a final concentration of 0.3 mM, and harvested the cells after an additional 2 h of incubation. Cells were resuspended in 10 mL buffer (20 mM Tris-HCl, 100 mM NaCl, pH 8.0) and disrupted by sonication. Inclusion bodies were collected via centrifugation at 9,500 × g for 1 h, washed twice with buffer (2% Triton X-100, 100 mM Tris-HCl, 100 mM NaCl, 2 M urea, pH 8.0), and incubated at room temperature for 4 h in 10 mL denaturation buffer (8 M urea, 20 mM Tris-HCl, 100 mM NaCl, 10 mM imidazole; pH 8.0) for denaturation. Solubilized proteins were recovered by centrifugation at 9,500 × g for 1 h, followed by passage of the supernatant through a syringe filter (0.45 μm). Proteins were loaded onto a Ni2+ column (1 mL HisTrap His-FF, GE Healthcare), which was subsequently washed with 10 mL denaturation buffer at a flow rate of 0.1 mL min⁻¹. Refolding of His6-SprA was induced by slowly (0.1 mL min⁻¹) replacing the denaturation buffer with 20 mL refolding buffer (20 mM Tris-HCl, 100 mM NaCl, 10 mM imidazole, 5 mM dithiothreitol, pH 8.0) through the application of a linear gradient. The proteins were then eluted with 5 mL elution buffer (20 mM Tris-HCl, 100 mM NaCl, 1 M imidazole, pH 8.0) at a flow rate of 1 mL min⁻¹. Proteins were concentrated via ultrafiltration (Amicon Ultra Centrifugal Filter Devices, Millipore), and the buffer was replaced with assay buffer (40 mM potassium phosphate, pH 8.0).

Proteolytic assays were conducted in a total volume of 25 μL at 30 °C by incubating proteins for 0–24 h. The assay buffer was composed of 40 mM potassium phosphate (pH 8.0). Subsequently, the assay mixtures were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins in the assay mixtures were as follows (pmol): 200 MBP-GlxR, 400 GlxR, 630 His6-SprA, and 110 bovine serum albumin (BSA).

Two-dimensional (2D)-PAGE and Enzyme Assays

2D-PAGE was conducted as previously described [30,34]. Before PAGE, protein extracts were solubilized in a rehydration buffer containing 9 M urea, 2 M thiourea, 4% (wt/vol) 3-[3-cholamidopropyl]-dimethylammonio]-1-propanesulfonate, 0.4 M Tris, 0.1 M (wt/vol) dithiothreitol, and 0.3% (vol/vol) IPG buffer (GE Healthcare) for 1 h. After rehydration, isoelectric focusing and second-dimension analyses were performed as described earlier [34]. Protein spots were visualized with Coomassie brilliant blue staining, and peptide analysis was performed by a commercial service (Proteinworks) via electrospray ionization mass spectrometry.

Cell lysates of C. glutamicum cells were prepared as described previously [6] and enzymatic activities of MS, ICL, and isocitrate dehydrogenase (ICDH) were determined as previously described [35].

Results

Isolation of Proteins Interacting with GlxR

To isolate protein(s) interacting with GlxR, we used a two-hybrid system with pSL500, which expresses GlxR, as the bait vector. Protein–protein interactions were screened based on the expression of his3 and adh4, which confer His+ and Str+, respectively. Introduction of the C. glutamicum genomic library into E. coli reporter cells harboring the bait vector allowed us to isolate five target clones exhibiting the His+ and Str+ phenotypes. Subsequently, the plasmids were isolated, and the DNA fragments in the “target” vector were sequenced. Two of the clones contained gene fragments that expressed peptide sequences, which turned out to be subtilisin-like serine protease (NCg0550) and Xaa-proline peptidase (NCg1430) [28,29]. To verify the interaction of the proteins with GlxR, we cloned the full-length open reading frames of NCg0550 and NCg1430 into the target vector, introduced them into reporter cells carrying the glxR-carrying bait vector, and monitored their growth on selection media. Only the cells carrying the NCg0550 gene showed growth on the medium (Figure 1). To quantify the protein–protein interaction, we measured the transcriptional level of the reporter gene him3 by RT-qPCR and determined it to be 11.3% compared with that of the positive control cells, which was set at 100%. The value observed in cells harboring empty vectors was 3.4%. Considering that the screening conditions may not have been physiologically ideal for inducing protein interactions (see Discussion), we tentatively concluded that GlxR specifically interacts with NCg0550-encoded protein. We then analyzed the gene further to see if the protein product interacted with GlxR and whether this interaction had physiological significance.

Analysis of NCg0550

The open reading frame of NCg0550 was 1,245 bps long and encoded a 43,143-Da protein composed of 414 amino acids. The
subtilisin-like serine proteases, of closely related *Mycobacterium tuberculosis* H37Rv, whereas the five serine proteases of the organism have been reported to show 36–47% identity with each other [39]. The encoded protein also showed 32% identity with the subtilisin-like serine protease (YaB, encoded by *ale*) of *Bacillus subtilis* [40]. Homologies with other subtilisin-like serine proteases were generally low (data not shown). Based on the homology with serine proteases, NCgl0550 was designated as *sprA* (serine protease A).

To study the role of *sprA* in *gixR*-mediated regulation, we first used RT-qPCR to analyze the expression profile of *sprA* during growth. The expression of *gixR* showed only marginal differences, whereas that of the *sprA* gene was maximal in the log phase and gradually decreased as the cells entered the stationary phase, suggesting that it plays a role in the active growth phase (Figure 2).

To determine the importance of *sprA*, we constructed a *C. glutamicum* *sprA* deletion mutant (Δ*sprA*) and a *sprA*-overexpressing strain (P180-*sprA*) and monitored their growth properties on minimal media. Internal deletion of a 494-bp fragment in *sprA* was verified by PCR (data not shown). The identity of the deleted gene was verified by complementary the Δ*sprA* strain with the plasmid pSL535 (i.e., pMT1-Δ*sprA*), which harbors the *sprA* gene with its own promoter; the complemented strain showed wild type-like growth (Figure 3A). Promoter P<sub>rpoB</sub> is known to overexpress the fused gene irrespective of the growth phase [31]. Overexpression of *sprA* (approximately 15-fold relative to that of the wild-type strain) was confirmed by RT-qPCR. As shown in Figure 3A, when grown on glucose minimal media, the Δ*sprA* and P<sub>180</sub>*sprA* strains exhibited reduced growth in the log phase relative to that of the wild-type strain, requiring longer incubation time to reach the stationary phase. In accordance with the expression data (see Figure 2), deletion of the gene did not significantly affect cell growth in the stationary phase. The differences in growth were further minimized when the cells were grown on acetate minimal medium (Figure 3B). Collectively, these data suggest a role for *sprA* in central metabolism during growth.

**In vitro Proteolysis of GixR by SprA**

Because SprA was isolated based on its capability to interact with GixR, we aimed to investigate protein-protein interactions in a purified system. The overexpressed MBP-GixR fusion protein was soluble and could be purified using a conventional approach without difficulty. However, the overexpressed His<sub>6</sub>-SprA fusion protein was insoluble due to the formation of inclusion bodies.

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### Table 1. Screened proteins and peptide sequences that interact with GixR.

| Screened sequence | Open reading frame with similarity | Annotated function | Frame<sup>1</sup> | ID<sup>2</sup> |
|-------------------|-----------------------------------|--------------------|-----------------|------------|
| GSTVYPHSDTLYSVARDFSHTLAEYMPGNQQLSAPSHIQAGLSPRGDGFASHMIRGRKNSV | NCgl0550 (sprA) | Subtilisin-like serine protease | In frame | 100 |
| GAENGANP8HGFSDRVRALNQGIDVIDIAQHTGFPPHYSDCTRTYVGQPDDADPRPQEFSLSSSSR | NCgl1430 | Xaa-Pro aminopeptidase | In frame | 93 |
| GSSSVLPWPAPPPGLPKVQKHKAKLAHHQKQIRGSQ | NCgl1535 | Pyrimidine reductase | Out of frame | 56 |
| GSHLPPPDDHLVWCQGQRP | NCgl2548 | Hypothetical protein | Out of frame | 46 |
| GSHYLPSSLSVLCSHGKTPKSSWHANPGLPFST | NCgl2510 | Pyridoxal-5’-phosphate-dependent aminotransferase | Out of frame | 28 |

<sup>1</sup>Reading frame of the cloned gene that produced the screened sequences.

<sup>2</sup>Identity (percent of matched amino acid sequence).

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Figure 2. Expression of glxR and sprA during growth. Cells were grown in glucose MCGB media, and mRNA levels were measured by RT-qPCR as described in the Materials and Methods. The data represent three independent experiments. Filled circles indicate the growth of C. glutamicum. Bars indicate mRNA levels of glxR (grey) and sprA (black), respectively. OD, optical density. doi:10.1371/journal.pone.0093587.g002

Therefore, we used a different purification approach, in which His6-SprA fusion protein was denatured with urea, bound to the column, and renatured on the column by slowly removing urea before elution. Once the protein was purified, it remained soluble (Figure 4A). However, during the purification process, partial degradation of His6-SprA was observed. Subsequently, we tested the stability of the purified His6-SprA by incubating it at 4°C or 30°C for 14 h. As shown in Figure 4B, the 48.2-kDa His6-SprA fusion protein underwent self-proteolysis during incubation at 4°C: SDS-PAGE revealed that fractions of purified SprA were digested to 27- to 35-kDa proteins (see Figure 4B, bracket 1). When the incubation was performed at 30°C, even smaller fragments were formed (see Figure 4B, bracket 2), indicating nearly complete digestion of His6-SprA. Although serine proteases are commonly synthesized as zymogens, which are converted to active enzymes via proteolytic cleavage at a particular peptide bond, no major identifiable protein band was detected in His6-SprA. In contrast, the 67.5-kDa MBP-GlxR fusion protein remained stable during purification and incubation; apparent self-proteolysis was not noted on performing SDS-PAGE (Figure 5, lanes 1 and 2) after a 24-h incubation period at 4°C or 30°C. After observing the proteolytic activity of His6-SprA, we tested it against MBP-GlxR. To minimize activity loss, we did not remove the protein fusion partners, such as MBP and His6, from each protein, eliminating additional purification steps. Interestingly, when the MBP-GlxR and His6-SprA fusion proteins were incubated together, the protein band corresponding to MBP-GlxR, as well as the band corresponding to SprA (Figure 5, lane 6), disappeared completely. However, a new 45-kDa protein band was detected by SDS-PAGE and remained stable. The M, of the protein (Figure 6A, lane 1) was nearly identical to that of purified MBP (see Figure 6A, lane 2). To elucidate its nature, we excised the band from the gel for quadrupole time-of-flight (Q-TOF) analysis. The amino acid composition of the band indicated that the protein product was MBP, suggesting that SprA is specific for its GlxR target. We performed additional analyses by incubating His6-SprA with BSA. As shown in Figure 6B, proteolysis of BSA was not observed, whereas self-proteolysis of His6-SprA was evident. Although we observed digestion of GlxR by SprA, the reaction was slow, taking a maximum of 24 h. This result could be due to the poor activity of SprA, which was purified using an on-column refolding procedure. The digestion of GlxR by SprA was only observable with fresh protein preparations. In addition, the fusion partners MBP and His6 may have hindered protein-protein interaction. Nevertheless, these data indicate that, although unstable, SprA acts on the globular form of GlxR and digests it with specificity. Furthermore, self-proteolysis of SprA may indicate the importance of controlling the availability of the protein in cells for GlxR and other proteins.

Effects of cAMP on the Proteolysis of GlxR by SprA

Intracellular cAMP levels in C. glutamicum are elevated during growth on glucose, especially in the early log phase, and low during growth on acetate. The DNA-binding activity of GlxR is also modulated by cAMP. Once SprA was found to specifically proteolyze GlxR, the effects of cAMP on the proteolysis were investigated, using native GlxR by removing MBP from MBP-GlxR. As shown in Figure 7 (lanes 1 and 2), purified GlxR remained stable during the incubation period at 4°C or 30°C. When GlxR was incubated in the presence of SprA at 30°C (see Figure 7, lane 7), both proteins completely disappeared, indicating complete proteolytic digestion. However, when the reaction was performed in the presence of cAMP, fractions of GlxR survived proteolysis (Figure 7, lane 8). Complete self-proteolysis of SprA was still evident in the reaction (Figure 7, lanes 5 and 8). These data demonstrate that, although GlxR is a substrate for SprA, it can still be protected from proteolysis by SprA depending on the physiological conditions of the cell.

Effects of sprA on the Expression of aceA, aceB, and icd

The genes aceA and aceB, which encode ICL and MS, respectively, are repressed by glxR in cells supplied with glucose as the sole carbon source. Therefore, one can speculate that C. glutamicum P180-glxR cells overexpressing the glxR gene may have depleted intracellular GlxR owing to proteolysis by SprA and may show derepression of aceA and aceB genes, even in cells supplied with glucose as the sole carbon source. Conversely, C. glutamicum cells grown on glucose have elevated levels of intracellular cAMP [6]. Therefore, as shown in Figure 7, SprA may be unable to proteolyze GlxR due to high intracellular cAMP levels in glucose-grown cells, resulting in the repression of aceA and aceB. Furthermore, the C. glutamicum ΔglxR strain, which has no intracellular GlxR, is known to have a severe growth defect [24,25], whereas the C. glutamicum P180-sprA strain, which is considered equivalent to the ΔglxR strain, showed reduced but reasonable growth on glucose minimal medium (see Figure 3A), suggesting that the intracellular GlxR might be intact. To determine whether the in vitro data of the present study agree with the reported observations for whole cells, we measured the ICL and MS activities from P180-glxR cells supplied with glucose as the carbon source. As shown in Table 2, the ICL and MS activities in P180-glxR cells were nearly comparable to those in the wild-type strain. These observations suggest that GlxR is almost intact in the cells, supporting the growth of the strain (see Figure 3A). Conversely, when acetate was supplied as the carbon source, as in the wild-type strain, derepression of glyoxylate bypass enzymes was observed in both the ΔglxR and P180-sprA strains (see Table 2). The observation that the level of derepression in the ΔglxR strain was comparable to that in the wild-type and P180-sprA strains may indicate that the amount of intracellular SprA is not important under those growth conditions. The results obtained with both ΔglxR and P180-sprA strains on glucose or acetate as the carbon source are in accordance with the in vitro data and suggest that the in vivo state of GlxR, the activity of which responds to cAMP, may
determine its fate as the substrate for the SprA protein (see Discussion).

Next, we measured the activity of isocitrate dehydrogenase (ICDH, encoded by \textit{icd}), which is a key enzyme for the tricarboxylic acid cycle and converts isocitrate to \(\alpha\)-ketoglutarate and CO\(_2\) with the concomitant release of reduced nicotinamide adenine dinucleotide phosphate. In \textit{C. glutamicum}, the enzyme is constitutively formed independent of the growth substrate [41,42]. Kim et al [6] have observed increased ICDH activity (twofold higher) in glucose-grown \textit{C. glutamicum} cells harboring multiple copies of \textit{glxR}, suggesting a positive role of \textit{glxR} in \textit{icd} expression, whereas no such effect was observed in acetate-grown cells. As shown in Table 2, the ICDH activity observed in the P\(_{180}\)-\textit{sprA} strain was only 32% of that of the wild-type strain, suggesting that the stimulatory effect of \textit{glxR} on \textit{icd} was abolished in the P\(_{180}\)-\textit{sprA} strain. In accordance with the finding by Kim et al [6], the enzyme activities of ICDH were found to be unaffected in acetate-grown cells regardless of the presence of the P\(_{180}\)-\textit{sprA} plasmid or \textit{D}\textit{sprA} mutation (see Table 2). The decrease in ICDH activity in glucose-grown cells was speculated to be due to the transcriptional control of \textit{icd} because activity was unaffected in acetate-grown cells. Although we do not know the cause of this result, we also observed a decrease in ICDH activity in \textit{D}\textit{sprA} cells grown with glucose as the sole carbon source. The activity was 50% of that in the wild-type strain (see Table 2). Decreases in ICDH activity in glucose-grown \textit{D}\textit{sprA} cells suggest the involvement of additional regulatory proteins (see Discussion). We hypothesized that the decrease in ICDH activity in P\(_{180}\)-\textit{sprA} and \textit{D}\textit{sprA} mutant cells contributed to the slower growth of the cells on glucose minimal medium (see Figure 3A). In support of this hypothesis, growth differences in the

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Growth characteristics of \textit{C. glutamicum} wild type and mutants. Cells were grown on glucose (A) or acetate (B) MCGC minimal media. The data represent three independent experiments. Symbols: •, wild-type \textit{C. glutamicum}; ○, \textit{C. glutamicum D}\textit{sprA}; ▲, \textit{C. glutamicum} harboring pSL509 (P\(_{180}\)-\textit{sprA}); △, \textit{C. glutamicum} harboring pSL535 (pMT1-\textit{sprA}).}
doi:10.1371/journal.pone.0093587.g003
\end{figure}
cells grown on acetate minimal medium were marginal (see Figure 3B). Similarly, the attenuation of ICDH activity decreases biomass yield and increases lysine production in lysine-producing C. glutamicum cells [43]. Collectively, these data suggest that sprA has unknown regulatory roles and that its involvement in glxR-mediated regulation may be more complex than anticipated.

Identification of Proteins under the Control of sprA

After investigating the role of sprA in general cell physiology (growth differences are shown in Figure 3, and differences in ICDH activity between P180-*sprA* and ΔsprA strains are in Table 2), we assumed that additional proteins might be affected in P180-*sprA* cells. To identify proteins with altered expression, we used 2D-PAGE analysis and compared the protein profiles of P180-*sprA* cells with those of wild-type cells. We chose P180-*sprA* cells because they exhibited a more severe growth defect than ΔsprA cells (Figure 4), showing that the decrease in spot intensity was caused by decreased transcription of the corresponding genes. Reduced transcription of the genes was also observed in ΔsprA cells (Figure 9), suggesting that the action of SprA was conveyed via transcriptional regulators, such as GlnR. Although genes in the GlnR regulon are mostly known from studies involving in silico genome analysis and in vivo and in vitro DNA-binding assays [20,21,23], the genes that we identified in the above analysis have not been listed among those in the GlnR regulon. However, the known functions of these proteins, such as those in energy and carbon metabolism (NCgl0049), nitrogen metabolism (NCgl0049), methylation reactions (NCgl0719), peptidoglycan biosynthesis (NCgl1267), as well as stress, starvation, and survival (NCgl0938) are associated with the functional category in which glxR plays a role. All of the data obtained suggest that SprA performs an important role not only in glxR-mediated gene regulation but also in other areas of cell physiology.

**Discussion**

In this study, we used a two-hybrid system to isolate the subtilisin-like serine protease SprA as a partner that interacts with GlnR. In the two-hybrid system, the bait (GlnR) and target (SprA) proteins are expressed as fusion proteins linked to the λcI repressor and RNA polymerase θ subunit, respectively. Interactions between the bait and target proteins stabilize the interaction of RNA polymerase with its weak promoter, resulting in the expression of *aadA* and *his3* reporter genes, which confer Strr and Hisr, respectively. The *in vivo* GlnR-SprA interaction was quantified using RT-qPCR, which measured his3 transcription, and determined to be low relative to that of the positive control. This decrease could be due to the proteolysis of GlnR with the interacting SprA, decreasing the transcription of the reporter genes. This assumption is highly supported by the observed GlnR proteolysis by SprA in a purified system. Self-proteolysis of SprA may have also contributed to the decrease in the reporter gene transcription. In addition, the binding of GlnR to SprA as a proteolytic substrate may not be strong enough to stabilize interactions between RNA polymerase and its promoter to allow transcription.

The finding that GlnR interacts with a protease is surprising because the common role of proteases is protein quality control, such as the degradation of damaged or unfolded proteins [49]. Subtilisin-like serine proteases are also generally secreted extracellularly to scavenge nutrients, although intracellular roles are also commonly found. Proteases are known to conduct critical regulatory functions via the proteolysis of regulators, enzymes, and other proteins [26,50]. Regulators that show either functional or structural similarity or both to GlnR also exist. For example, the global transcription factor FNR, which plays a role in anaerobiosis

**Figure 4. Purification and self-proteolysis of SprA.** Purified His6-SprA (A) was incubated at 4°C or 30°C for 14 h as described in the Materials and Methods (B). The arrows indicate purified His6-SprA. The self-proteolysis of His6-SprA is shown in brackets 1 and 2 of B. M, molecular weight marker. doi:10.1371/journal.pone.0093587.g004
in *E. coli*, is controlled by proteolysis [51,52] as well as the oxidative stress response regulator Spx in low-GC Gram-positive bacteria [53]. The global regulator Mlc, which is a transcriptional repressor of sugar-metabolizing enzymes and uptake systems in *E. coli*, is also regulated by proteolysis [54]. A protease system is also involved in the cellular turnover of FixK2, which is a CRP-like transcription factor that controls the endosymbiotic lifestyle of *Bradyrhizobium japonicum* [55]. In *C. glutamicum*, the Clp complex and FtsH protease are involved in nitrogen control through action on GlnK [56,57]. In addition, *C. glutamicum* FtsH protease has been implicated in the regulation of energy and carbon metabolism as well as in amino acid biosynthesis [58]. Therefore, it is not surprising to find SprA proteolyzing the global regulator GlxR with specificity. A serine protease MycP1 from *M. tuberculosis*, a close relative of *C. glutamicum*, also performs a novel role with defined substrate specificity [59].

In general, serine proteases show broad substrate specificity. Although many function as general proteases for unfolded substrates, some cleave only one substrate. In addition, many prokaryotic serine proteases show domain architectural diversity and thus function in an organism-specific manner [38]. Several observations suggest that *sprA* performs a novel role in *C. glutamicum*: (a) Both deletion and overexpression of the gene resulted in a retarded growth phenotype, indicating a global role for the gene. (b) The transcription of *sprA* showed an atypical temporal pattern, suggesting a specialized role for the gene. Typically, most subtilisin genes are expressed at low levels during the log growth phase [60]. *M. tuberculosis* *myc* genes are also expressed constitutively [39]. (c) SprA was not homologous to other subtilisin-like serine proteases. (d) Complete self-proteolysis of SprA is unique and suggests that tight control of SprA availability is important owing to its regulatory role in cell physiology. (e) Although *myc* genes are present in closely related *M. tuberculosis*, only one homologous gene (*sprA*) is found in *C. glutamicum*, suggesting a novel role.

Although we initially isolated SprA as a protein that specifically interacted with GlxR, its regulatory role now appears to be much more complex than we expected. First, the P180-*sprA* strain showed no lethal phenotype, indicating that GlxR was still active in the cell but reduced cell growth, which suggests that other cellular functions were affected. In addition, the proteins identified with 2D-PAGE are not known to be included in the GlxR regulon, suggesting that putative GlxR-binding motifs are absent in the regulatory region of the identified genes. Furthermore, the activity of ICDH was severely affected in ΔsprA and P180-*sprA* cells. *Icd*, encoding ICDH, showed no *glxR*-binding motif in its promoter and regulatory regions and is excluded from the GlxR regulon. However, this gene is still regulated by *glxR*, as noted in our study and by Kim et al [6], suggesting unknown regulation in central carbon metabolism. In *E. coli*, the *icd* gene has two different promoters and is controlled by a complex regulatory system involving the global regulatory proteins ArcA, Fnr, and Cra [61,62]. The expression of *icd* in *E. coli* is controlled by carbon and oxygen availability; its expression decreased during anaerobic growth and increased in the presence of poor carbon sources, such
Figure 6. Proteolytic specificity of His₆-SprA. Proteolytic assays were performed as described in the Materials and Methods. Proteins were incubated at 4°C or 30°C for 24 h, analyzed on a 15% SDS-PAGE gel, and then visualized by staining with Coomassie brilliant blue G250. Lanes 1 and 2 (A) show digested His₆-SprA (identical to lane 6 in Figure 5) and purified MBP, respectively. (B) Digestion of bovine serum albumin (BSA) with His₆-SprA. Molecular weights are shown in kDa, and M denotes the protein marker. doi:10.1371/journal.pone.0093587.g006

Figure 7. Effects of cAMP on in vitro digestion of GlxR by His₆-SprA. Protein purification and proteolytic assays were performed as described in the Materials and Methods. Proteins were incubated at 4°C or 30°C for 20 h, analyzed on a 15% SDS-PAGE gel, and then visualized by staining with Coomassie brilliant blue G250. Molecular weights are shown in kDa. M, molecular weight markers; and MBP, maltose-binding protein. doi:10.1371/journal.pone.0093587.g007
as acetate. In this type of control, ArcA and Fnr act negatively, and Cra exerts positive control. Analogous to this, our results suggest a complex regulatory mechanism for the *C. glutamicum* icd gene as well.

The activity of GlxR is modulated by cAMP. In all the cases studied, the *in vitro* binding of GlxR to its target binding sites is strictly dependent on the presence of cAMP. Unlike the levels in *E. coli*, intracellular cAMP levels in *C. glutamicum* are reported to be elevated during growth on glucose and low during growth on acetate [6]. Therefore, cells grown on glucose may have GlxR in its holo form; that is, GlxR may exist as a cAMP-bound protein. However, as shown in Figure 7, SprA may not act on the holo form of GlxR. We observed proteolysis of GlxR by SprA only in the absence of cAMP, suggesting that only the apo form of GlxR could serve as the substrate for SprA. The *in vitro* binding of GlxR to its target DNA requires the addition of cAMP to the assay mixture, probably to convert the GlxR into its cAMP-bound holo form, which is considered active and protected from proteolysis by SprA, thereby performing its function by repressing glyoxylate bypass genes. As a result, ICL and MS activities are minimal in glucose-grown *D sprA* and *P 180-sprA* strains, as shown in Table 2.

The reasonable growth of the P 180-*sprA* strain on glucose minimal medium (see Figure 3A) suggests that intracellular GlxR is mostly intact and supports the above-mentioned hypothesis. Conversely,

### Table 2. Enzyme activities of isocitrate lyase (ICL), malate synthase (MS), and isocitrate dehydrogenase (ICDH) in cell extracts of *C. glutamicum* cells*

| Carbon source | Strains    | Phenotype | Specific activity, μmol min⁻¹ mg⁻¹ | ICL | MS | ICDH |
|---------------|------------|-----------|-----------------------------------|-----|----|------|
| Glucose       | AS019E12   | Wild type | 0.116                             | 0.092 | 0.008 |
|               | HL1385     | ΔsprA     | 0.098                             | 0.096 | 0.004 |
| Acetate       | HL1389     | P₁₈₀-sprA | 0.110                             | 0.098 | 0.003 |
|               | AS019E12   | Wild type | 0.428                             | 0.506 | 0.008 |
|               | HL1385     | ΔsprA     | 0.389                             | 0.508 | 0.009 |
|               | HL1389     | P₁₈₀-sprA | 0.391                             | 0.434 | 0.008 |

*The enzymes were induced by growing *C. glutamicum* AS019E12 cells to the late log phase on MCGC minimal medium. Cells were harvested, disrupted, and assayed for the activity as described in the Materials and Methods. The activities represent one of three independent experiments.

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![Figure 8. Identification of protein affected in sprA-overexpressing *C. glutamicum* (P₁₈₀-sprA). 2D-PAGE was performed as described in the Materials and Methods. A total of 150 μg of protein was loaded onto each gel. The identity of each protein spot was determined by electrospray ionization mass spectroscopy.](doi:10.1371/journal.pone.0093587.g008)
in acetate-grown cells, GlxR probably exists in its apo form; that is, it does not bind to cAMP owing to low intracellular cAMP levels. If cAMP is absent in the cells, the DNA-binding activity of GlxR remains minimal, thereby deerepressing the glyoxylate bypass genes. Under this condition, the overexpression of sprA (P_{sprA}sprA) or deletion of the gene (ΔsprA) has no significant effect on the expression of glyoxylate bypass genes, as shown in Table 2. In wild-type cells, residual intracellular GlxR in its apo form can be further proteolyzed by SprA and cleared from the cell, preventing residual GlxR from performing any regulatory role (see below). Thus, our hypothesis that only the apo form of GlxR, which is not expressed during growth (see Figure 2), but as conditions fluctuate, GlxR, which once served a useful function, likely requires clearance from the cell at times. The primary goal of SprA may be to remove GlxR when it is no longer needed. As mentioned above, cAMP probably plays a key role in GlxR-SprA interaction and the stimulation of proteolysis. The constitutive expression of glxR, temporal expression of the sprA gene (see Figure 2), and self-proteolysis of SprA to remove itself after use (see Figure 4) support this hypothesis.

In conclusion, we isolated SprA as an interacting partner for GlxR. SprA preferentially functions in the log phase and mediates the proteolysis of GlxR with specificity. In addition, sprA appears to play additional regulatory roles in general cell physiology. The self-proteolytic activity of SprA suggests that the immediate and timely removal of SprA is also important to ensure precise regulatory control.

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Author Contributions

Conceived and designed the experiments: EH HL. Performed the experiments: EH. Analyzed the data: EH YK HL. Contributed reagents/materials/analysis tools: HL. Wrote the paper: EH HL. Construction library for BacterioMatch II hybrid system: JP.

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