Lactoferricin B Inhibits the Phosphorylation of the Two-Component System Response Regulators BasR and CreB*§

Yu-Hsuan Ho‡, Tzu-Cheng Sung‡, and Chien-Sheng Chen‡§

Natural antimicrobial peptides provide fundamental protection for multicellular organisms from microbes, such as Lactoferricin B (Lfcin B). Many studies have shown that Lfcin B penetrates the cell membrane and has intracellular activities. To elucidate the intracellular behavior of Lfcin B, we first used Escherichia coli K12 proteome chips to identify the intracellular targets of Lfcin B. The results showed that Lfcin B binds to two response regulators, BasR and CreB, of the two-component system. For further analysis, we conducted several in vitro and in vivo experiments and utilized bioinformatics methods. The electrophoretic mobility shift assays and kinase assays indicate that Lfcin B inhibits the phosphorylation of the response regulators (BasR and CreB) and their cognate sensor kinases (BasS and CreC). Antibacterial assays showed that Lfcin B reduced E. coli’s tolerance to environmental stimuli, such as excessive ferric ions and minimal medium conditions. This is the first study to show that an antimicrobial peptide inhibits the growth of bacteria by influencing the phosphorylation of a two-component system directly. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.014720, 1–10, 2012.

Multicellular organisms have developed several types of defense systems against various environmental microbes. One mechanism of defense is antimicrobial peptides (AMPs)\(^1\); these natural peptides exist in a wide range of the multicellular organisms including animals and plants, which suggests that the AMPs play very important roles in basic prokaryotes. This system is typically composed of a histidine sensor kinase and a response regulator; the histidine kinase is presented in the membrane and activated by an environmental stimulus. The intracellular response regulator controls the gene expression or physiologic action of the cell. In E. coli, ~30 histidine kinases and 34 response regulators have been suggested to exist (3, 4), and the roles of these systems are quite various, which enables the microbes to overcome the diverse challenges they encounter in their natural habitats (5).

AMPs have been an important challenge for microorganisms to overcome. In Salmonella, the sensor kinase PhoQ in the inner membrane of cells is activated by sensing the AMPs and then transmits the signal to its cognate response regulator PhoP (6). PhoP then makes the cells less susceptible to AMPs by activating the genes that modify the cell surface (6–9). In addition, the PmrA-PmrB system in Pseudomonas aeruginosa has also been found to regulate the resistance to cationic AMPs such as polymyxin B and E, cattle indolicidin, and LL-37 (10). In this system, the sensor kinase acts as an important element to recognize AMPs and then activates the cognate response regulator to start the defense mechanism against the membrane active AMPs. However, no study has been reported on the attack of AMP on the TCS system.

Some AMPs have been proven to penetrate the cell membrane and affect the intracellular targets within the cells (11–13); Lfcin B is one of these AMPs (14–18). Some studies have indicated that Lfcin B leads to the depolarization of the cell membrane and does not lyse the cells (19); Lfcin B has been shown to inhibit the macromolecular synthesis of cells, which suggests that the intracellular targets of Lfcin B may exist (12, 19, 20).

Using E. coli proteome microarrays, we discovered that Lfcin B strongly bound two TCS response regulators (21, 22). We further characterized the relationships between these two proteins with Lfcin B by in vitro and in vivo analysis. The mechanism by which Lfcin B influences the normal function of these two TCS response regulators was also elucidated.

From the ‡Graduate Institute of Systems Biology and Bioinformatics, National Central University, Jhongli 32001, Taiwan

Received September 28, 2011, and in revised form, December 2, 2011

Published, MCP Papers in Press, December 2, 2011, DOI 10.1074/mcp.M111.014720

1 The abbreviations used are: AMP, antimicrobial peptides; LB, Luria broth; COG, clusters of orthologous groups; TCS, two component system.

For bacteria, some of these AMPs can be recognized by two-component systems (TCSs) to protect the integrity of the bacterial cell membranes; TCSs are very common conserved signal transduction mechanisms among prokaryotes. This system is typically composed of a histidine sensor kinase and a response regulator; the histidine kinase is presented in the membrane and activated by an environmental stimulus. The intracellular response regulator controls the gene expression or physiologic action of the cell. In E. coli, ~30 histidine kinases and 34 response regulators have been suggested to exist (3, 4), and the roles of these systems are quite various, which enables the microbes to overcome the diverse challenges they encounter in their natural habitats (5).

AMPs have been an important challenge for microorganisms to overcome. In Salmonella, the sensor kinase PhoQ in the inner membrane of cells is activated by sensing the AMPs and then transmits the signal to its cognate response regulator PhoP (6). PhoP then makes the cells less susceptible to AMPs by activating the genes that modify the cell surface (6–9). In addition, the PmrA-PmrB system in Pseudomonas aeruginosa has also been found to regulate the resistance to cationic AMPs such as polymyxin B and E, cattle indolicidin, and LL-37 (10). In this system, the sensor kinase acts as an important element to recognize AMPs and then activates the cognate response regulator to start the defense mechanism against the membrane active AMPs. However, no study has been reported on the attack of AMP on the TCS system.

Some AMPs have been proven to penetrate the cell membrane and affect the intracellular targets within the cells (11–13); Lfcin B is one of these AMPs (14–18). Some studies have indicated that Lfcin B leads to the depolarization of the cell membrane and does not lyse the cells (19); Lfcin B has been shown to inhibit the macromolecular synthesis of cells, which suggests that the intracellular targets of Lfcin B may exist (12, 19, 20).

Using E. coli proteome microarrays, we discovered that Lfcin B strongly bound two TCS response regulators (21, 22). We further characterized the relationships between these two proteins with Lfcin B by in vitro and in vivo analysis. The mechanism by which Lfcin B influences the normal function of these two TCS response regulators was also elucidated.
Lfcin B Inhibits the Phosphorylation of BasR and CreB

EXPERIMENTAL PROCEDURES

Fabrication of the E. coli K12 Proteome Chips—For the studies of the bacterial proteome, we have constructed the E. coli K12 proteome microarray (22). In short, the E. coli K12 ASKA library (23) was first incubated with 2 × Luria Broth (LB) medium containing 30 μg/ml chloramphenicol in 96 DeepWell™ plates (Nunc) at 37 °C overnight. Then, the overnight cultures were diluted with 2 × LB to an OD600 value of 0.1. When the cells grew to an OD600 value of 0.7–0.9, isopropyl β-D-thiogalactoside was added to cells to induce protein expression at 37 °C for 3.5 h. The cultures were harvested by centrifugation at 2240 × g for 5 min at 4 °C, and the pellets were stored at −80 °C before purification.

To purify the proteins, the frozen cell pellets were thawed on ice and resuspended in 40 μl lysis buffer consisting of 50 mM NaH2PO4, 300 mM NaCl, 30 mM imidazole, and 0.1% Tween 20, pH 7.5. After the purified proteins BasR and CreB were diluted in kinase buffer (50 mM NaH2PO4, 300 mM NaCl, 30 mM imidazole, and 0.1% Tween 20, pH 8), they were then stored at 20 °C. To observe the binding affinity of Lfcin B for BasS and CreB for CreC, we labeled the purified proteins BasS and CreC first with DyLight 549 according to the instruction manual (Pierce, Rockford, IL). The labeled proteins BasS and CreC were then diluted with the binding buffer (50 mM Tris-HCl, pH 8.0, 37 °C, 50 mM KCl, and 10 mM MgCl2) to 5 μM. The 0.1 μg of Lfcin B and Crecinop P1 were added onto the chip and incubated at 37 °C for 30 min in BioMixer (Capital Bio). After a wash with TBS, labeled BasS and CreC were added to each well and incubated in BioMixer at 37 °C for 30 min. Finally, the chips were washed with TBS and scanned by a laser scanner LuxScan (Capital Bio).

The Kinase Assays—The purified proteins BasR and CreB were diluted to 0.05 mg/ml in TBS and immobilized on a 96-well plate (Nunc) at room temperature for 2 h. The Lfcin B and Crecinop P1 were then diluted in TBS to a final concentration of 0.017 μg/μl. The peptides were added into the 96-well plate and incubated at 37 °C for 30 min. After the incubation and a wash with TBS, the purified proteins BasS and CreC were diluted in kinase buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, and 10 mM MgCl2) to 5 μM, and the phosphorylation reaction was started by adding ATP to a final concentration of 0.5 mM. The protein solutions of BasS and CreC were distributed in the 96-well plate and incubated at 37 °C for 30 min. Each well was washed with TBS, and the PhosPho;Enzyme Enrichment Kit (27–32) was used to stain the phosphorylated BasR and CreC for 1 h. Finally, the fluorescence intensity was measured by the Synergy 2 (BioTek) with an excitation wavelength of 543 nm and an emission wavelength of 580 nm.

The Antibacterial Assays—The antibacterial activity of Lfcin B in different medium was observed for testing the specificity. Approximately 107 to 108 CFU/ml of E. coli BW25113 was transferred into a 96-well plate (Nunc). Each well contained 100 μl LB broth with or without 1.5 mM of ferric ions) or minimal medium. Minimal medium was prepared with 6 g/liter NaH2PO4, 3 g/liter K2HPO4, 0.5 g/liter NaCl, 0.12 g/liter MgSO4 7 H2O, 0.4 g/liter KH2PO4, and 0.5 g/liter CaCl2, and the carbon resource was mainly from 5 g glucose/liter. A 0.22 micron filter was used to sterilize the minimal medium. A total of 50 μg/ml of Lfcin B and the control peptide (Crecinop P1) were added to the plate. The OD600 of each well was recorded every 20 min by the Synergy 2 (BioTek).

The Gap Sequence Analysis of 34 E. coli Response Regulators—The 34 E. coli response regulator sequences were processed by the online tool MEME (33), which identified the common motifs within the input sequences. Because the similarities within the response regulators are high, the MEME output showed some highly conserved regions among the 34 response regulators; we only retrieved the sequences that were not highly conserved. After we retrieved the gap sequences of all 34 response regulators, the ClustalX2 was used to align the sequences and analyze the results.
RESULTS

The E. coli Proteome Chip Assays and Bioinformatics Analysis—We employed a high-throughput E. coli protein microarray (22) to systematically identify the possible target proteins of Lfcin B. We chose DyLight™ 649 labeled streptavidin as our signal reporter for biotinylated Lfcin B and Cecropin P1. Because all E. coli proteins have histidine tags, DyLight™ 549 labeled anti-His antibody binds to the histidine tags of all of the proteins on the chips. Thus, the signal of the labeled anti-His antibody indicated the relative protein amount for all the proteins printed on the chips. Biotinylated AMPs were first used to probe the E. coli proteome chips. DyLight™ 649 labeled streptavidin and DyLight™ 549 labeled anti-His antibody were then used to probe the chips. After the washing steps, the chip was scanned and analyzed. The analyzed image signal was output as a text file for signal normalization and positive hit selection. Each signal was normalized with ProCAT (24). The distribution of the signal was fit to a normal distribution, and the positive hits were selected based on a local cut-off, which was defined as two standard deviations above the signal mean for each spot. After the initial selection, the top 2% of proteins were then defined as final hits for Lfcin B. These 30 identified proteins were specific to Lfcin B, and all of them were defined as negative hits for Cecropin P1 under the same threshold.

Initially, we classified our top 30 candidates based on the categories of the NCBI COGs database. The COGs of E. coli MG1655 consist of 25 categories, and each protein is assigned to one or more categories. A total of 3,559 proteins were classified by COGs. The top 30 proteins were mapped based on the COG categories. The summary of this analysis is shown in Table I. These 30 proteins showed enrichment in three categories: translation (fold enrich: 4.59), transcription (fold enrich: 3.76) and function unknown (fold enrich: 3.83); their p values were all less than 0.01. These results indicate that Lfcin B may affect the transcription and translation of microbes. Lfcin B has been observed to inhibit the synthesis of DNA, RNA, and macro-molecules (12). Thus, our results are in agreement with previous findings.

The top 30 proteins are displayed in Fig. 1. Two TCS response regulators, BasR and CreB, were found within the top 30 protein hits, which suggests that they strongly bound to Lfcin B. In this study, we focused on investigating the interactions between these two response regulators and Lfcin B; Cecropin P1 was used as the negative control. The mechanism by which Cecropin P1 disrupts the cell membrane of bacteria has been fully characterized. In contrast, Haukland et al. (20) have proved that Lfcin B can penetrate the cell membrane without disruption; yet, Cecropin P1 has only been found to surround the membrane.

A chip image comparison between Lfcin B and Cecropin P1 is shown in Fig. 2. Lfcin B showed a strong binding signal for BasR and CreB; yet for Cecropin P1, no binding was observed between Cecropin P1 and these two proteins. This result indicates that Lfcin B selectively bound to BasR and CreB in comparison with Cecropin P1. Thus, BasR and CreB are specific targets of Lfcin B.

Lfcin B Does Not Inhibit the DNA Binding Activity of BasR and CreB—Typically, the E. coli response regulators have two domains: one is the receiver domain, and the other is the DNA binding domain (34). We applied EMSA to observe whether Lfcin B affects the DNA binding ability of BasR and CreB. For BasR, we used a known promoter, pmrH, that is recognized by BasR to observe whether the binding between pmrH and

| Category        | Total number of proteins | Hit number | p value | Odds ratio |
|-----------------|--------------------------|------------|---------|------------|
| J: Translation  | 188                      | 5          | 0.008   | 4.59       |
| K: Transcription| 321                      | 7          | 0.005   | 3.76       |
| S: Function unknown | 315                  | 7          | 0.005   | 3.83       |

Fig. 1. The top 30 protein hits that were identified from the chip assays. To examine the identified protein hits in more detail, we classified the 30 proteins based on their functions. Two two-component system response regulators, BasR and CreB, were present in the top 30 hits.
Lfcin B Inhibits the Phosphorylation of BasR and CreB

BasR is influenced by Lfcin B (25). Fig. 3 displays the interactions between Lfcin B, BasR, and its promoter, and also between Lfcin B, CreB, and its promoter. An obvious band shift was observed when BasR was added to the pmrH promoter; this indicates that BasR bound to the pmrH promoter, which caused pmrH and BasR to move more slowly than the pmrH without BasR (Fig. 3A, lane A). A dramatically band shift was also observed at the top of the gel when Lfcin B was added to pmrH with BasR (Fig. 3A, lane B). We also added the negative control Cecropin P1 to pmrH with BasR. No band was observed at the top of the gel (Fig. 3A, lanes A and C). These findings indicate that Lfcin B, BasR, and pmrH formed a large complex and that this large complex could not migrate through the gel. Lfcin B appears to bind strongly to the receiver domain of BasR, and this binding does not cause BasR to lose it ability to recognize pmrH.

We used a known promoter, radC (26), that is recognized by CreB to test the interactions between Lfcin B, CreB, and its promoter. The same phenomenon was observed for CreB, as shown in Fig. 3B. Similar to the results for BasR, a band was observed at the top of the gel once CreB was incubated with Lfcin B. Lfcin B bound to CreB without affecting the binding between CreB and radC (Fig. 3B, lanes A and C). This result also indicates that Lfcin B binds to the receiver domain of CreB and not to the DNA binding domain.

Lfcin B Inhibits the Interactions Between CreB and CreC—The receiver domain is also the contact domain of BasR and CreB for their sensor partners, BasS and CreC (34). Thus, we examined whether Lfcin B influences the binding between these two response regulators and their cognate sensor kinases. We printed purified BasR, CreB, and the negative controls, YabO and FtsY, into epoxy framed chips. BasS and CreC were first labeled with fluorescence. The testing wells in a chip were first incubated with Lfcin B or Cecropin P1, and then labeled BasS and CreC were incubated in all of the wells. As shown in Fig. 4, when the well was first incubated with the Lfcin B, the signal dropped dramatically. The negative control Cecropin P1 only slightly decreased the signal intensity, which was possibly caused by the nonspecific binding of Cecropin P1 to CreB. This result indicates that Lfcin B binds to the receiver domain of CreB and occupies the CreC binding site. It should be noted that binding between BasR and BasS was difficult to detect via this chip assay, which suggests that the interaction between BasS and BasR is transient or very weak.

Lfcin B Blocks the Phosphorylation of BasR and CreB—In TCSs, the histidine kinase senses the environmental stimulus and then phosphorylates the cognate response regulator at the aspartate residue of the receiver domain (34). The phosphorylated response regulator has a higher DNA binding affinity than the unphosphorylated response regulator does (35). In fact, phosphorylation is also required for the response regulator to undergo the proper conformational change to interact with RNA polymerase and initiate the transcriptional activity (36). Our EMSA and binding assay results all indicate that Lfcin B may interact with the receiver domains of BasR and CreB, which suggests that Lfcin B may inhibit the phosphorylation from BasS to BasR and from CreC to CreB. To test this hypothesis, we immobilized BasR and CreB separately in the 96-well plates and then phosphorylated them with their cognate sensor kinases BasS and CreC. The Pro-Q Diamond phosphoprotein stain was used to stain phosphorylated BasR and CreB. We were able to observe whether the phosphorylation degree of BasR and CreB was influenced by Lfcin B. Fig. 5 displays the phosphorylation state of BasR and CreB after they were incubated with or without Lfcin B or Cecropin P1. Once the immobilized BasR and CreB were first incubated with Lfcin B, the degree of the phosphorylation of BasR and CreB dropped significantly.

Until now, it has been very clear that the mechanism by which Lfcin B influences the response regulators of BasR and CreB is via the inhibition of the phosphorylation communication of BasR to BasS and CreB to CreC.

Lfcin B Reduces E. coli’s Tolerance of a High Ferric Ion Concentration—To observe whether the functions of BasR and CreB are affected by Lfcin B, we conducted an in vivo
antibacterial assay. TCSs enable bacteria to grow normally when they encounter certain threats in their environments. The TCS response regulators act as necessary elements to control gene expression for responding to various conditions. Hagiwara et al. (5) reported that the response regulator BasR is crucial for *E. coli* to survive when cells encounter a high concentration of ferric ions. If BasR is targeted by Lfcin B, the ability of bacteria to respond to excessive ferric ions may be limited. Thus, we examined the antibacterial capability of Lfcin B in an environment of excessive ferric ions. Fig. 6 demonstrates the antibacterial activity of Lfcin B in different media. As shown in Figs. 6A and 6B, the inhibition capability of Lfcin B was stronger when the bacteria were grown in medium with an excess of ferric ions than when they were grown in the normal LB medium. For the control peptide, Cecropin P1 exhibited the same antibacterial activity under the different media. This result indicates that Lfcin B targeted BasR and thus the ability of BasR to receive the signal from BasS was altered; therefore, the mechanism mediated by BasR in response to excessive ferric ions was influenced, and this eventually lead to a growth defect in the cells.

**Lfcin B Reduces *E. coli*'s Tolerance in Minimal Media**—Avison et al. (26) showed that CreB is a global regulator that greatly influences metabolic control in *E. coli* and is activated in minimal medium. Nikel et al. (37) reported that the mutant strain of creB had a low glucose uptake rate and an abnormal biomass condition. Because CreB is a response regulator that is activated when bacteria are grown in minimal medium, we observed the antimicrobial capability of Lfcin B in minimal media to determine whether Lfcin B may exhibit an enhanced effect on bacteria. The antibacterial ability of Lfcin B against *E. coli* in minimal media is shown in Fig. 6C. In the

**FIG. 3.** Lfcin B does not inhibit the DNA binding activity of BasR and CreB. A, The gel patterns in lanes A and C were similar, which suggests that BasR bound to its promoter, pmrH, and Cecropin P1 did not bind to BasR. However, in lane B, there appears to be a large complex that formed and could not migrate through the gel. These results indicate that Lfcin B, BasR, and pmrH became a large complex, and the ability of BasR to recognize the promoters was not affected. B, Similarly, CreB was strongly recognized by Lfcin B, and the ability of CreB to capture the promoter radC was not influenced. A large complex also formed between Lfcin B, CreB, and radC (lane B). Therefore, it is clear that Lfcin B bound to the receiver domains of BasR and CreB so that the DNA binding domains of BasR and CreB retained their function.

**FIG. 4.** Lfcin B inhibits the binding of the response regulator CreB and its sensor kinase CreC. CreB and two negative controls, YabO and FtsY, were printed on the epoxy 12-well framed chip. Each well contained identical spots including CreB, yabO, and ftsY. Lfcin B, Cecropin P1, and PBS (negative control) were first incubated in the corresponding wells, and then the labeled protein CreC was incubated in all of the wells. The signal intensity decreased dramatically when the CreB protein was exposed to Lfcin B. This result indicates that Lfcin B bound to CreB and inhibited the ability of CreC to recognize CreB. In addition, the two negative controls showed weak signal intensities, which indicated that the binding of CreC to CreB was specific.

**Lfcin B Inhibits the Phosphorylation of BasR and CreB**

Molecular & Cellular Proteomics 11.4 10.1074/mcp.M111.014720–5
minimal media, Lfcin B completely inhibited the bacterial growth, and Cecropin P1 did not show significant growth inhibition. In LB medium (Fig. 6), the antibacterial activity of Lfcin B was not as obvious as it was in minimal medium. This result indicates that Lfcin B has greatly enhanced antibacterial ability in minimal medium and suggests that the function of CreB was altered by Lfcin B and that the bacteria had difficulty utilizing the glucose as a carbon source for survival in minimal media.

To summarize, these results suggest that Lfcin B penetrated the cell membrane and attacked the two response regulators BasR and CreB, which limited the ability of the bacteria to respond to the irregular environment.

DISCUSSION

Generally, multicellular organisms coexist with many types of microbes by maintaining a dynamic balance between immune defense and microbe colonization. Perturbations of this balance will contribute to the development of disease. AMPs are important for maintaining the necessary symbiotic relationships. For example, insects do not have a complicated immune system that includes as antibodies and lymphocytes; however, insects are able to survive in the presence of microbes by utilizing AMPs as a major defense system (38). Some TCSs are able to activate the defense system by sensing AMPs. These findings suggest that TCS acts as a defense system for bacteria to overcome attack from the host AMPs.

In this study, our chip methodology provides a reliable platform for studying the mechanism of Lfcin B and also reveals novel findings between Lfcin B and TCS. This is the first study to show that AMPs attack the TCS of bacteria. In this study, Lfcin B did not affect the DNA binding ability of the unphosphorylated response regulators, BasR and CreB. Instead, Lfcin B was observed to attack the receiver domains of BasR and CreB to inhibit their phosphorylation activity. In fact, the unphosphorylated response regulators are still able to recognize promoters (39); however, it is unlikely for microbes to over-produce the response regulators to activate or repress transcription. Phosphorylation is also important for the response regulators to change their conformation to interact with RNA polymerase and initiate transcription (40). Thus, the ability to phosphorylate is essential for BasR and CreB to activate gene expression in their natural habitats. Our findings indicate that Lfcin B blocks the communications of response regulators to sensor kinases (BasR-BasS and CreB-CreC). The phosphorylation signal is not able to be passed from the sensor kinases to the response regulators. This leads to the insufficient reactive actions of bacteria in response to environmental stress.

The human gastrointestinal tract is full of various microbes and contains ~300–500 bacterial species (41). The environment of the gastrointestinal tract contains excess metals that permeabilize the bacterial cell surface and results in a lethal effect on the microbes (42, 43). To overcome this stress, E. coli has adapted the BasRS system to survive in this stressful environment. In response to the excess ferric ions in the environment, BasRS mediates the lipopolysaccharide (LPS) modification of the cell surface and prevents the damage from Fe (III) (5, 44). Interestingly, Lfcin B is a pepsin-digested peptide of bovine lactoferrin and is also present in the human gastrointestinal tract (45). In this study, we showed that Lfcin B inhibits the phosphorylation capability of BasR and also has enhanced antibacterial activity in the environment of excessive ferric ions. These results indicate that Lfcin B penetrates the bacterial membrane and selectively targets the receiver domain of BasR of the microbes to limit the number of microbes by blocking the phosphorylation activity between BasR and BasS. Thus, the signal of excessive ferric ions that is sensed by BasS cannot be passed to BasR, which inhibits the growth of the microbes in the intestines by limiting the capability for LPS modification. Moreover, a study also indicated that this Fe (III) mediated killing mechanism is similar to that exhibited in certain AMPs, such as polymyxin B (25).
Once the BasR is targeted by Lfcin B, the microbes may be unable to react to the attack by certain AMPs, such as polymyxin B, because the antibacterial mechanism of polymyxin B is very similar to that of Fe(III). This phenomenon also suggests that cooperation may exist among several AMPs to protect the host from bacteria. Lfcin B may serve to shut down the defense system of microbes to facilitate other AMPs to attack the microbes.

In addition to BasR, we also identified that Lfcin B strongly recognizes another TCS response regulator, CreB. CreBC has been identified as an important metabolic regulator in a recent study (37). Although no direct relation between the CreBC of microbes and the metabolism within the gastrointestinal tract has been reported, previous studies have demonstrated that CreBC responds to the change of the carbon sources that are utilized by microbes (39). Various types of microbes colonize
the human intestine to compete for carbon resources (46). Thus, we proposed that the CreBC system may be important for microbes to utilize the frequently changing carbon sources. Carbon may also be crucial for some microbes to colonize in their habitats (46). In this study, we showed that Lfcin B targeted the response regulator CreB and inhibited the phosphorylation between CreB and CreC, and thereby dramatically eliminated the growth of E. coli in the minimum medium. This observation indicated that Lfcin B plays an important role for microbes to compete for the carbon resources in the intestines.

In addition to the perspective on metabolism, a recent report indicated that the activation of the CreBC system is related to the tolerance of E2 colicin (47). Colicins are protein antibiotics produced by specific strains of E. coli and are toxic for some bacterial strains. The role of colicin for E. coli to successfully colonize in the gastrointestinal tract is also critical because colicin is toxic to other bacterial strains (48). Thus, once CreB is attacked by Lfcin B, the ability of bacteria to resist the attack from E2 colicin may be limited.

These results demonstrated the diverse and important functions mediated by the CreBC system. CreBC controls the central role for carbon metabolism of bacteria and is also involved in the tolerance of some antibacterial agents. Thus, the attack of Lfcin B on CreB may lead to comprehensive effects on microbes in their niches.

TCS has drawn much attention from antibiotic-design researchers to target the TCS common domains for therapeutic intervention (49–51). One important factor that makes TCS an attractive target is that the signal transduction method of bacterial TCS is by histidine/aspartate, which is distinct from the serine/threonine system that eукarvoty adopt. This makes the bacterial TCS an attractive target for the design of antibacterial drugs. Until now, several inhibitors that target the histidine kinases have been introduced; however, most of them have shown poor selectivity for TCS (52, 53). Many reports also suggested that the response regulators may be good targets because the sensor kinases may not be completely inactive because of the complex signal transduction network, and some small molecule phosphate donors will also contribute to the phosphorylation of the response regulators (49, 54, 55).

To uncover the Lfcin B-binding motifs of BasR and CreB, we used MEME (33) to identify the highly conserved motifs among all 34 response regulators of E. coli (Fig. S1A). All these response regulators share many conserved features in their N-terminal receiver domains. Because Lfcin B only specifically bound to BasR and CreB, the Lfcin B-binding motif of BasR and CreB may lie in the less conserved regions of the 34 response regulators, such as in residues 15–35 and 55–85. After analyzing these sequences by ClustalX2, we identified a consensus sequence between BasR and CreB in residues 55–85. This motif is L-P-V-L-[FI]-L-T-A, a small hydrophobic and leucine rich motif. It is located near the phosphorylation site of BasR and CreB, which indicates that once Lfcin B targets this region, it probably affects the phosphorylation of BasR and CreB. Our kinase assays have shown that Lfcin B affects the phosphorylation of BasR and CreB. We also observed similar motifs in Salmonella, Shigella and Yersinia (supplemental Fig. S1B). Lfcin B was confirmed to inhibit the growth of bacteria including Salmonella and E. coli (56–58). Additionally, Lfcin B was able to reduce the adhesion to and invasion of host cells by Yersinia (59). However, the homologous examination of BasR and CreB in probiotics, including Bifidobacterium and Lactobacillus (supplemental Fig. S1C), showed that this motif was not observed, suggesting that Lfcin B may have less effect on the probiotics in the intestine. In fact, it was reported that Lactoferrin, the precursor of Lfcin B, has a dramatic inhibitory effect on certain pathogens while probiotics such as Bifidobacterium and Lactobacillus were not affected by Lactoferrin (60). Furthermore, the combination of Lactoferrin with probiotics has been confirmed to have enhanced antibacterial effect in the clinical trials designed to overcome the infections caused by Helicobacter pylori (61).

In summary, the relationship between TCS and AMP is bidirectional; certain well-known TCSs are able to sense specific AMPs and trigger the protection mechanisms to defend against the attack of AMPs; however, in this study, we uncovered a novel interaction between TCS and AMP: AMP attacks TCS. This mutual relationship perfectly illustrates the balance between the host immune system and the response system of microbes.

Acknowledgments—We thank Dr. Hirotada Mori and colleagues for constructing the E. coli ASKA library.

This work was supported by the National Science Council, Taiwan (NSC98-2627-M-008-004 and 99-2627-M-008-003) and partly by the Veterans General Hospitals and University System of Taiwan (VGHUST99-G4–1), Cathy General Hospital (99CGH-NCU-A3), Landseed Hospital (NCU-LSH-100-A-012), the Ministry of Education of Taiwan, the grant Plan of Developing Top Universities And Research Centers, the Aim for the Top University Project, National Central University, and the Center for Dynamical Biomarkers and Translational Medicine (CDBTM).

This article contains supplemental Fig. S1.

§ To whom correspondence should be addressed: Graduate Institute of Systems Biology and Bioinformatics, National Central University, 300, Jhongda Rd., Jhongli 32001, Taiwan. Tel.: +886-3-4227151; ext. 36103; E-mail: cchen103@gmail.com.
44. Chamnongpol, S., Dodson, W., Cromie, M. J., Harris, Z. L., and Groisman, E. A. (2002) Fe(III)-mediated cellular toxicity. *Mol. Microbiol.* 45, 711–719

45. Leitch, G. J., and Ceballos, C. (2009) A role for antimicrobial peptides in intestinal microsporidiosis. *Parasitology* 136, 175–181

46. Fabich, A. J., Jones, S. A., Chowdhury, F. Z., Cernosek, A., Anderson, A., Smalley, D., McHargue, J. W., Hightower, G. A., Smith, J. T., Austert, S. M., Leatham, M. P., Lins, J. J., Allen, R. L., Laux, D. C., Cohen, P. S., and Conway, T. (2008) Comparison of carbon nutrition for pathogenic and commensal Escherichia coli strains in the mouse intestine. *Infect. Immun.* 76, 1143–1152

47. Cariss, S. J., Constantinidou, C., Patel, M. D., Takebayashi, Y., Hobman, J. L., Penn, C. W., and Avison, M. B. (2010) YieJ (CbrC) mediates CreBC-dependent colicin E2 tolerance in Escherichia coli. *J. Bacteriol.* 192, 3329–3336

48. Gillor, O., Giladi, I., and Riley, M. A. (2009) Persistence of colicinogenic Escherichia coli in the mouse gastrointestinal tract. *BMC Microbiol.* 9, 165

49. Stephenson, K., and Hoch, J. A. (2002) Two-component and phosphorelay signal-transduction systems as therapeutic targets. *Curr. Opin. Pharmacol.* 2, 507–512

50. Kenney, L. J. (2002) Structure/function relationships in OmpR and other winged-helix transcription factors. *Curr. Opin. Microbiol.* 5, 135–141

51. Shrivastava, R., Ghosh, A. K., and Das, A. K. (2007) Probing the nucleotide binding and phosphorylation by the histidine kinase of a novel three-protein two-component system from Mycobacterium tuberculosis. *FEBS Lett.* 581, 1903–1909

52. Hillard, J. J., Goldschmidt, R. M., Licata, L., Baum, E. Z., and Bush, K. (1999) Multiple mechanisms of action for inhibitors of histidine protein kinases from bacterial two-component systems. *Antimicrob. Agents Chemother.* 43, 1693–1699

53. Stephenson, K., and Hoch, J. A. (2004) Developing inhibitors to selectively target two-component and phosphorelay signal transduction systems of pathogenic microorganisms. *Curr. Med. Chem.* 11, 765–773

54. Gotoh, Y., Eguchi, Y., Watanabe, T., Okamoto, S., Doi, A., and Utsumi, R. (2010) Two-component signal transduction as potential drug targets in pathogenic bacteria. *Curr. Opin. Microbiol.* 13, 232–239

55. Bourret, R. B. Receiver domain structure and function in response regulator proteins. *Curr. Opin. Microbiol.* 13, 142–149

56. Yamauchi, K., Tomita, M., Giehl, T. J., and Ellison, R. T., 3rd. (1993) Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. * Infect. Immun.* 61, 719–728

57. Orsi, N. (2004) The antimicrobial activity of lactoferrin: current status and perspectives. *Biometals* 17, 189–196

58. Bellamy, W., Takase, M., Wakabayashi, H., Kawase, K., and Tomita, M. (2009) Persistence of colicinogenic Escherichia coli in the mouse gastrointestinal tract. *BMC Microbiol.* 9, 165

59. Tian, H., Maddox, I. S., Ferguson, L. R., and Shu, Q. Influence of bovine lactoferrin on selected probiotic bacteria and intestinal pathogens. *Biometals* 23, 593–596

60. Artym, J., and Zimecki, M. (2005) [The role of lactoferrin in the proper development of newborns]. *Postepy Hig Med Dosw* 59, 421–432

61. Tang, Z., Yin, Y., Zhang, Y., Huang, R., Sun, Z., Li, T., Chu, W., Kong, X., Li, L., Geng, M., and Tu, Q. (2009) Effects of dietary supplementation with an expressed fusion peptide bovine lactoferricin-lactoferrampin on performance, immune function and intestinal mucosal morphology in piglets weaned at age 21 d. *Br. J. Nutr.* 101, 998–1005

In order to cite this article properly, please include all of the following information: Ho, Y.-H., Sung, T.-C., and Chen, C.-S. (2012) Lactoferricin B Inhibits the Phosphorylation of the Two-Component System Response Regulators BasR and CreB. *Mol. Cell. Proteomics* 11(4):M111.014720. DOI: 10.1074/mcp.M111.014720.