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Solution Structure of the Peptidoglycan Binding Domain of *Bacillus subtilis* Cell Wall Lytic Enzyme CwlC: Characterization of the Sporulation-Related Repeats by NMR[†,‡]

Masaki Mishima,[§] Toshio Shida,[||] Kazuto Yabuki,[§] Ken-ichi Kato,[§] Junichi Sekiguchi,[||] and Chojiro Kojima*,[§]

Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma 630-0101, Japan, and Department of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Tokida, Japan

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ABSTRACT: *Bacillus subtilis* CwlC is a cell wall lytic *N*-acetylmuramoyl-L-alanine amidase that plays an important role in mother-cell lysis during sporulation. The enzyme consists of an N-terminal catalytic domain with C-terminal tandem repeats. The repeats [repeat 1 (residues 184−219) and repeat 2 (residues 220−255)] are termed CwlCr. We report on the solution structure of CwlCr as determined by multidimensional NMR, including the use of 36 $^{h3}J_{NC}{}'$-derived hydrogen bond restraints and 64 residual $^{1}D_{NH}$ dipolar couplings. Two tandem repeats fold into a pseudo-2-fold symmetric single-domain structure consisting of a $\beta\alpha\beta\beta\alpha\beta$-fold containing numerous contacts between the repeats. Hydrophobic residues important for structural integrity are conserved between the repeats, and are located symmetrically. We also present NMR analysis of the circularly permuted repeat mutant of CwlCr. Secondary structure content from the chemical shifts and hydrogen bonds derived from $^{h3}J_{NC}{}'$ show that the mutant folds into a structure similar to that of the wild type, suggesting that the repeats are exchangeable. This implies that conserved hydrophobic residues are crucial for maintaining the folding of the repeats. While monitoring the chemical shift perturbations following the addition of digested soluble peptidoglycan fragments, we identified two peptidoglycan interaction sites of CwlCr at the edges of the protein symmetrically, and they are located ∼28 Å from each other.

Bacillus subtilis utilizes a set of enzymes capable of hydrolyzing the peptidoglycan layer of its own cell wall (*1*−*3*). Some of these peptidoglycan hydrolases can trigger cell lysis and are termed autolysins (*1*). These have been implicated in several important cellular processes, including cell wall turnover, cell separation, competence, and motility (*1*, *4*, *5*). *N*-Acetylmuramoyl-L-alanine amidases, which constitute the major autolysins, cleave the amido bond between the lactyl group of an *N*-acetylmuramic acid residue and the α-amino group of an alanine residue (*6*). These enzymes were initially purified and characterized from *B. subtilis* (*7*−*12*).

CwlC amidase, an *N*-acetylmuramoyl-L-alanine amidase, is secreted from sporulating *B. subtilis* cells (*13*) and can hydrolyze vegetative cell walls and spore peptidoglycan in vitro. Furthermore, a *cwlB cwlC* double mutant is resistant to mother-cell lysis during the late stage of sporulation (*13*). CwlC is a 27 kDa protein composed of 255 amino acids and consists of modular structural components comprising an N-terminal catalytic domain and two C-terminal tandem repeat sequences (Figure 1A,B). Application of the cell wall hydrolysis assay using a C-terminal repeat truncation mutant of CwlM, a homologous *Bacillus* enzyme whose sequence is 72% identical to that of CwlC, indicated that cell wall preference was impaired (*14*), and that the CwlC C-terminal repeats were required for efficient catalytic activity (*15*). These findings implied that the C-terminal repeat sequences play an important role in peptidoglycan binding. The Pfam database indicated that the C-terminal repeat sequences belong to a “sporulation-related repeat” family (pfam05036) (*16*), one of a conserved sequence family. This repeat is found in a tandem manner in many proteins involved in sporulation and cell division. However, direct biochemical data showing an interaction between peptidoglycan and the repeat have not been published.

In this study, we present the solution structure of the C-terminal repeat sequences, designated CwlCr, consisting of repeat 1 (residues 184−219) and repeat 2 (residues 120−255), by heteronuclear multidimensional NMR. On the basis of the well-defined structure, detailed structural pictures were described. The peptidoglycan interaction sites of CwlCr were identified using NMR, representing the first report showing that the sporulation-related repeats directly bind peptidoglycan itself. Furthermore, the structure of the circularly permuted repeat mutant was also characterized by NMR. Interestingly, the mutant adopted a similar folded structure, implying that the hydrophobic residues conserved between the repeats were key residues involved in folding.

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[‡] The atomic coordinates of the ensemble of CwlCr structures have been deposited in the Protein Data Bank as entry 1X60.

* To whom correspondence should be addressed. Telephone: +81-743-72-5571. Fax: +81-743-72-5579. E-mail: kojima@bs.naist.jp.

[§] Nara Institute of Science and Technology.

[||] Shinshu University.

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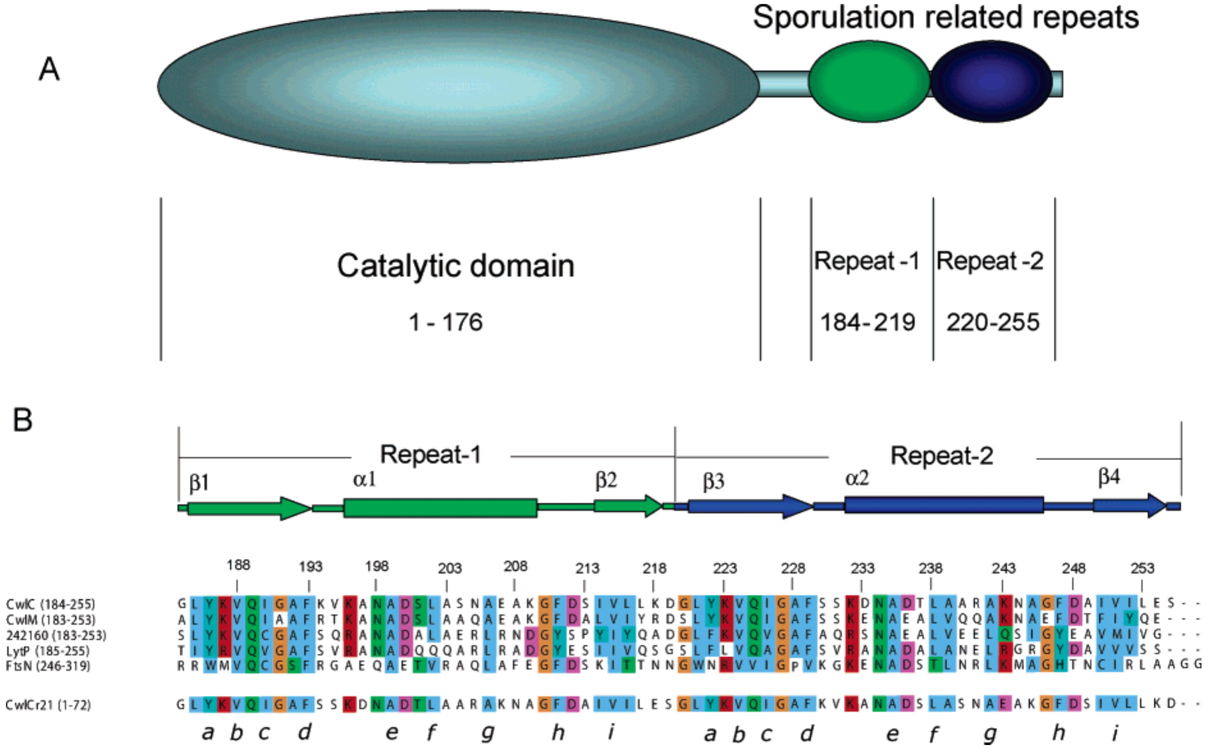

FIGURE 1: (A) Domain structure of CwlC. N-Terminal catalytic domain and C-terminal repeat sequences are schematically indicated. (B) Multiple-sequence alignment of sporulation-related repeats of cell wall lytic amidase. The secondary structure elements of CwlCr are shown schematically at the top. *a*−*i* denote the conserved hydrophobic residues. CwlCr, CwlM, 242160, LytP, and FtsN represent partial sequences of *B. subtilis* CwlC, *B. subtilis* CwlM, *Bacillus halodurans* C-125 sporulation mother cell wall hydrolase (NCBI accession number NP_242160), *Bacillus* sp. LytP, and *E. coli* FtsN, respectively. The first and last sequence numbers are indicated in parentheses. The repeat sequence of CwlCr21(1−72) is swapped, and residues 1−36 and 37−72 correspond to residues 220−255 and 184−219 of the wild type, respectively. Sequence alignment was initially obtained from a BLAST database search, realigned using ClustalW (*54*), and annotated using JALVIEW (*55*).

EXPERIMENTAL PROCEDURES

Sample Preparation. CwlCr was expressed in *Escherichia coli* M15 harboring a plasmid encoding CwlCr. Uniform labeling of proteins with ^{15}N or ^{15}N and ^{13}C was achieved using M9 minimal medium containing $^{15}NH_4Cl$ and [$^{13}C_6$]-glucose as the sole sources of nitrogen and carbon, respectively. Cells were grown at 37 °C, and protein expression was induced using isopropyl 1-thio-β-D-galactopyranoside when the A_{660} was 0.5. Cells were harvested 4 h following induction. Harvested wet cells were resuspended in 50 mM HEPES buffer (pH 7.5) containing 400 mM KCl and 0.1 mM EDTA. The suspension was lysed by sonication and ultracentrifuged, and the supernatant was loaded onto DEAE-Sepharose. The flow-through fraction was collected and purified using a Hitrap chelating column (Amersham Biosciences), charged with Ni ions, and eluted stepwise using imidazole. The hexahistidine tag was removed by specific cleavage using enterokinase. The protein was further purified by being passed through a Hitrap-S cation-exchange column (Amersham Biosciences). The homogeneity and identity of the purified protein were examined by SDS−PAGE and N-terminal analysis (M492 Perkin-Elmer), respectively. Protein concentrations were estimated using the calculated molar absorption coefficient at 280 nm ($\epsilon_{280} = 2.68 \times 10^3$ M^{-1} cm^{-1}). The expression and purification of the circularly permuted repeat mutant, CwlCr21, were performed as described above.

NMR Spectroscopy and Determination of the Structure of CwlCr. Purified CwlCr was dissolved in 50 mM potassium phosphate buffer (pH 6.5) containing 20 mM KCl and 0.1 mM EDTA in either a 95% H_2O/5% ^2H_2O mixture or 99.8% ^2H_2O. The final concentration of the protein was 1.7 mM. NMR spectra were acquired at 30 °C on a Bruker AVANCE 500 instrument equipped with a cryogenic probe and a Bruker DRX800 NMR spectrometer. Chemical shifts were referenced to 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). For the purposes of collecting residual dipolar coupling restraints, nonionic liquid crystalline medium was used, consisting of 50 mM potassium phosphate buffer (pH 6.9), 20 mM KCl, 0.1 mM EDTA, 10% ^2H_2O, and a 5% $C_{12}E_5$PEG [*n*-dodecyl penta(ethylene glycol)]/hexanol mixture with a surfactant-

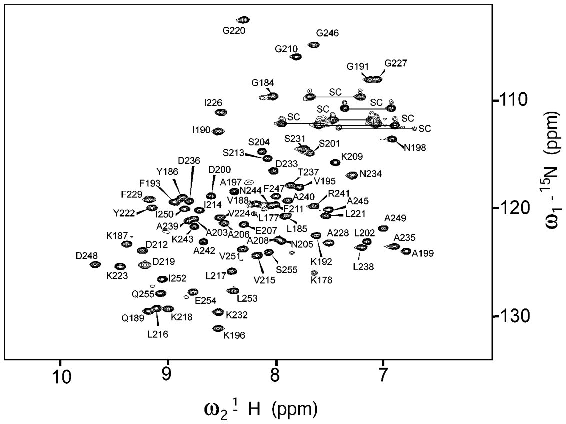

FIGURE 2: ^{1}H−^{15}N HSQC spectrum of CwlCr. The spectrum was obtained with 1.7 mM CwlCr at pH 6.5 and 30 °C. Assignments of the backbone amide groups are labeled. The SC label indicates side chain peaks of asparagine or glutamine residues.

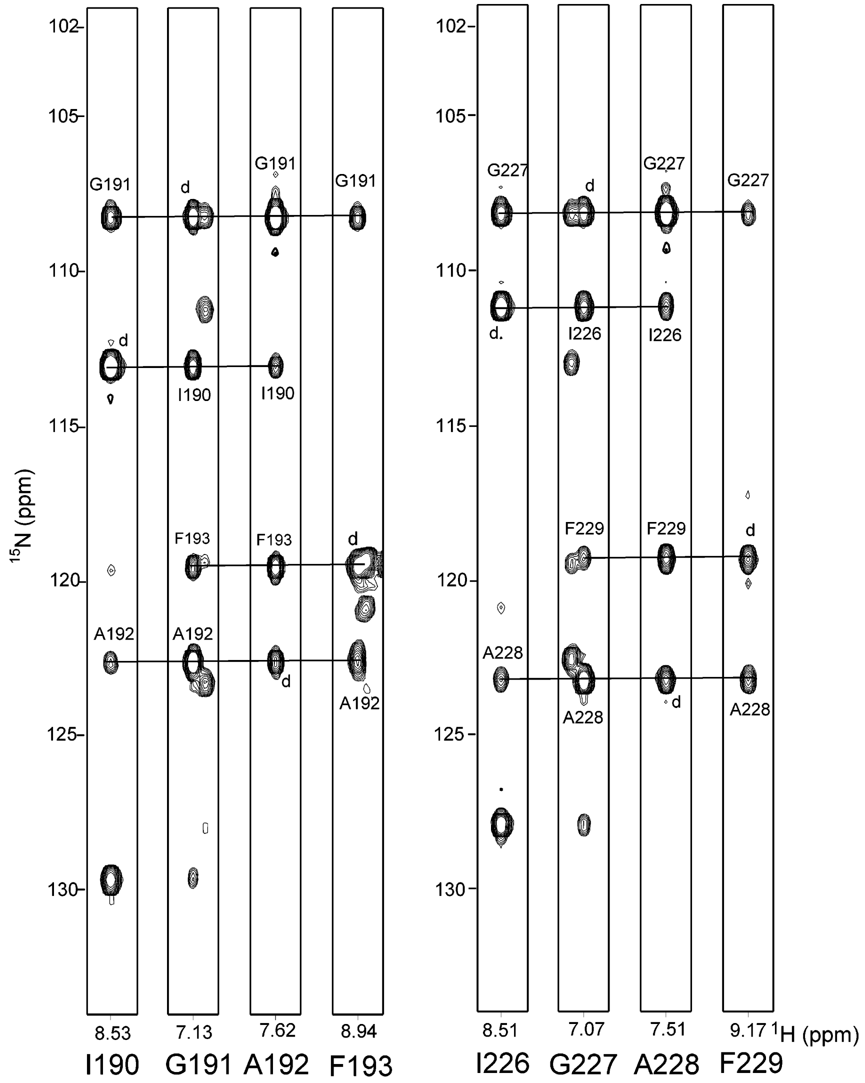

FIGURE 3: Selected $\omega_3(^1H)/\omega_1(^{15}N)$ strips from a 3D (H)N(CO-TOCSY)NH spectrum of CwlCr. The spectrum was acquired on a Bruker Avance 500 spectrometer equipped with a Cryogenic probe over the course of 30 h using a 1.7 mM solution of ^{13}C- and ^{15}N-labeled CwlCr at pH 6.9 and 30 °C. The strips are taken from slices at the backbone amide ^{15}N (F_2) frequency of each residue ranging from I190 to F193 (left) and from I226 to F229 (right). The experiment correlates the amide ^{1}H (F_3) and ^{15}N (F_2) chemical shift of each residue to the ^{15}N (F_1) chemical shift of the neighboring two or more residues in a relay manner. Sequential connectivity is shown by solid horizontal lines, and diagonal peaks are denoted with d.

to-alcohol ratio of 0.96 (*17*). This medium induced a deuterium splitting of ~15 Hz on the AVANCE 500 instrument. All multidimensional NMR spectra were acquired in a phase sensitive mode employing a States−TPPI or Rance−Kay method (*18*). The water flip-back method (*19*) was applied in several experiments that use amide proton magnetization. Shifted sine-bell window functions were applied to the NMR data prior to zero-filling and Fourier transformation. Mirror-image or forward−backward linear prediction was also used. All spectra were processed with the NMRPipe package (*20*), and analyzed using Sparky (*21*). ^{1}H, ^{13}C, and ^{15}N assignments were mainly obtained from standard multidimensional NMR methods (*22*, *23*), HNCACB, HN(CO)CACB, HN(CA)CO, and HNCO for main chain assignments and C(CO)NH, H(CCO)NH, HCCH-TOCSY, and four-dimensional (4D) HC(CO)NH (*24*) for side chain assignments. Any ambiguity relating to the sequential assignments was solved by a (H)N(CO-TOCSY)NH experiment (*25*). This experiment was slightly modified from the original sequence, wherein the first INEPT polarization transfer period (^{15}N to ^{13}C′) was set to 54 ms, rather than the original 108 ms, since long delays caused significant sensitivity loss for CwlCr. Consequently, the adiabatic decoupling scheme for the aliphatic region was converted to simple decoupling using a Gaussian-cascade 3 pulse. The methyl groups of all Leu and Val residues were assigned in a stereospecific manner using a ^{13}C−^{1}H constant time HSQC spectrum of a randomly 15% ^{13}C-enriched protein sample (*26*). Most of the aromatic ^{1}H resonances were assigned by two-dimensional (2D) NOESY[1] on an unlabeled protein sample. Hydrogen bond restraints were derived from $^{h3}J_{NC'}$ couplings observed in the $^{h3}J_{NC'}$HNCO experiment (*27*, *28*). Interproton distances were derived from 2D NOESY, three-dimensional (3D) ^{15}N-edited NOESY-HSQC, 4D ^{13}C/^{15}N-edited HMQC-NOESY-HSQC, and 3D ^{13}C-edited NOESY-HSQC spectra (*22*). Backbone dihedral angles were evaluated from vicinal coupling constants ($^3J_{HNHA}$) obtained from an HNHA experiment (*22*). Additionally, dihedral ϕ and ψ

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[1] Abbreviations: NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single-quantum correlation spectroscopy; rms, root-mean-square.

Table 1: Structural Statistics for CwlCr[a]

total no. of distance constraints	1206
long-range ($\|i - j\| > 4$)	361
middle-range ($\|i - j\| = 2$, 3, or 4)	232
short-range ($\|i - j\| = 1$)	276
intraresidue	265
hydrogen bond constraints	36 × 2
no. of dihedral constraints	
ϕ, φ	58, 58
χ_1	18
residual dipole couplings $^1D_{NH}$	63
rms deviation from experimental constraints[b]	
distance (Å)	0.0291 ± 0.0005
angle (deg)	0.78 ± 0.03
$^1D_{NH}$ (Hz)	0.63 ± 0.01
rms deviation from idealized covalent geometry	
bonds (Å)	$0.0023 \pm 5 \times 10^{-5}$
angles (deg)	0.434 ± 0.004
impropers (deg)	0.330 ± 0.006
CNS energy terms (kcal/mol)	
E_{bond}	6.1 ± 0.2
E_{angle}	62 ± 1
E_{imp}	9.7 ± 0.4
E_{vdw}[c]	-193 ± 8
PROCHECK Ramachandran plot (residues 185−254)	
residues in most favored regions (%)	95.4
residues in additional allowed regions (%)	4.2
residues in generously allowed regions (%)	0.1
residues in disallowed regions (%)	0.3
rms deviation of mean structure derived from 30 calculated structures	
backbone atoms (residues 185−254) (Å)	0.16
all heavy atoms (residues 185−254) (Å)	0.52

[a] These statistics comprise the ensemble of the 30 lowest-energy structures obtained from 100 starting structures. Structure calculations were performed using CNS version 1.1. [b] None of these structures exhibited distance violations of >0.4 Å, dihedral angle violations of >5°, or residual $^1D_{NH}$ dipolar coupling violations of >2 Hz. [c] E_{vdw} is the Lennard-Jones energy of CNS energy terms.

angles derived from TALOS were also used in the final refinement step (*29*). The χ_1 rotomer of the side chains was estimated from HNHB and HN(CO)HB experiments (*30*, *31*). Residual $^1D_{NH}$ couplings were extracted from the difference in *J* splittings measured for isotropic and anisotropic samples. The *J* splittings for ^{15}N−^{1}H were obtained from 2D ^{1}H−^{15}N IPAP experiments performed in an interleaved manner (*32*). All NMR spectra were analyzed using Sparky (*21*).

CYANA version 1.05 with the CANDID protocol was used for the purposes of structural restraint collection (*33*). Finally, an ensemble of 100 CwlCr structures was calculated using CNS version 1.1 with residual $^1D_{NH}$ couplings by a standard simulated annealing protocol (*34*). Initial estimation for the axial component of the molecular alignment tensor (D_a) and the rhombicity (R) were obtained on the basis of the structure calculated with CYANA using PALES (*35*). These values were optimized in an iterative manner, using the structures calculated by CNS. The final values of D_a and R were 6.28 Hz and 0.32, respectively. The final 30 lowest-energy ensemble structures were checked by PROCHECK-NMR (*36*), and graphics were created with MOLMOL (*37*).

NMR Spectroscopy of CwlCr21. Multidimensional NMR spectroscopy for CwlCr21 was performed essentially as described for CwlCr. Briefly, HNCACB, HN(CO)CACB, HN(CA)CO, and HNCO experiments for main chain resonance assignments were performed on the DRX800 instrument (*22*, *23*). HN(CO-TOCSY)NH and $^{h3}J_{NC'}$HNCO experiments were performed on the AVANCE 500 instrument (*25*, *27*, *28*).

Chemical Shift Perturbation Experiments. Soluble digested peptidoglycan was prepared by enzymatic digestion of purified peptidoglycan from vegetative cells of *B. subtilis* with intact CwlC. The reaction that was employed was carried out essentially according to the published procedure (*15*). Recombinant CwlC enzyme and purified peptidoglycan were prepared essentially as previously described (*15*). Chemically synthesized building blocks of peptidoglycan, (1) diaminopimelic acid (DL-α,ϵ-diaminopimelic acid), (2) Ala-D-γ-Glu-Lys-D-Ala-D-Ala, (3) GlcNAc [*N*-acetyl-D-(+)-glucosamine], and (4) GlcNAcMurNAcAlaGln [*N*-acetyl-D-glucosaminyl-β-(1,4)-*N*-acetylmuramyl-L-alanyl-D-iso-glutamine], were purchased from SIGMA or Wako.

^{1}H and ^{15}N amide resonance changes in uniformly ^{15}N-labeled CwlCr were monitored following the addition of a large excess of additives to 20 mM HEPES buffer (pH 6.9) containing 0.1 mM CwlCr, 20 mM KCl, 1 mM EDTA, and 5% ^2H_2O. The ratio of the diaminopimelic acid, Ala-D-γ-Glu-Lys-D-Ala-D-Ala, GlcNAc, and GlcNAcMurNAcAlaGln to CwlCr was 100:100:100:10. CwlC-digested peptidoglycan could be dissolved in an aqueous solution up to 0.5 mg/mL, and the perturbation experiment was performed with 0.5 mg/mL digested peptidoglycan. MALDI-TOF MS analysis of the major fragments of the digested products showed multiplets around 158×10^2. The ^{1}H−^{15}N HSQC spectra were obtained at 30 °C. For each cross-peak, the normalized weighted average shift difference, $\delta_{ave}/\delta_{max}$, was calculated (*38*, *39*). The weighted average shift difference, δ_{ave}, was calculated as $[\delta_{^1H}{}^2 + (\delta_{^{15}N})^2/25]^{1/2}$, where $\delta_{^1H}$ and $\delta_{^{15}N}$ represent the difference in parts per million between the free and perturbed chemical shifts. The δ_{max} value represents the maximum observed weighted average shift difference. Changes in signal intensity were also evaluated using the ratio of the intensity difference caused by perturbation and the reference spectrum, $(I_{ref} - I_{per})/I_{ref}$, where I_{ref} and I_{per} represent the signal intensity of the reference and perturbed spectrum, respectively.

RESULTS AND DISCUSSION

Structure Determination. Isotopically labeled recombinant CwlCr (residues 177−255) was overexpressed in *E. coli* and purified by affinity tag and ion-exchange chromatography. The protein obtained was able to bind peptidoglycan (data not shown). The ^{15}N−^{1}H HSQC spectrum in Figure 2 shows highly dispersed cross-peaks, suggesting that CwlCr adopts a stable tertiary structure in solution.

NMR resonance assignments were obtained by performing double- and triple-resonance NMR experiments using ^{15}N-labeled and ^{15}N- and ^{13}C-labeled protein samples. Almost all of the main chain resonance assignments were obtained from the HNCACB, HN(CO)CACB, HN(CA)CO, and HNCO spectra. In this process, the sequential walk was confirmed by the well-dispersed amide ^{15}N and ^{1}H chemical shifts obtained from the 3D (H)N(CO-TOCSY)-NH experiment, where the magnetization transfer pathway was not through space but through the bonds, with $^1\mathrm{HN} \rightarrow {}^{15}\mathrm{N}(t_1) \rightarrow {}^{13}\mathrm{C}' \rightarrow$ isotropic mixing $\rightarrow {}^{13}\mathrm{C}' \rightarrow {}^{15}\mathrm{N}(t_2) \rightarrow {}^1\mathrm{HN}(t_3)$. This experiment provided the correlation between amide N($n \pm 1$) and N(n) and HN(n). $\omega_3(^1\mathrm{H})/\omega_1(^{15}\mathrm{N})$ strips taken through the ^{15}N diagonal peaks in the $\omega_1(^{15}\mathrm{N})/\omega_2(^{15}\mathrm{N})$ plane displayed sequential amide−amide connectivity (Figure 3).

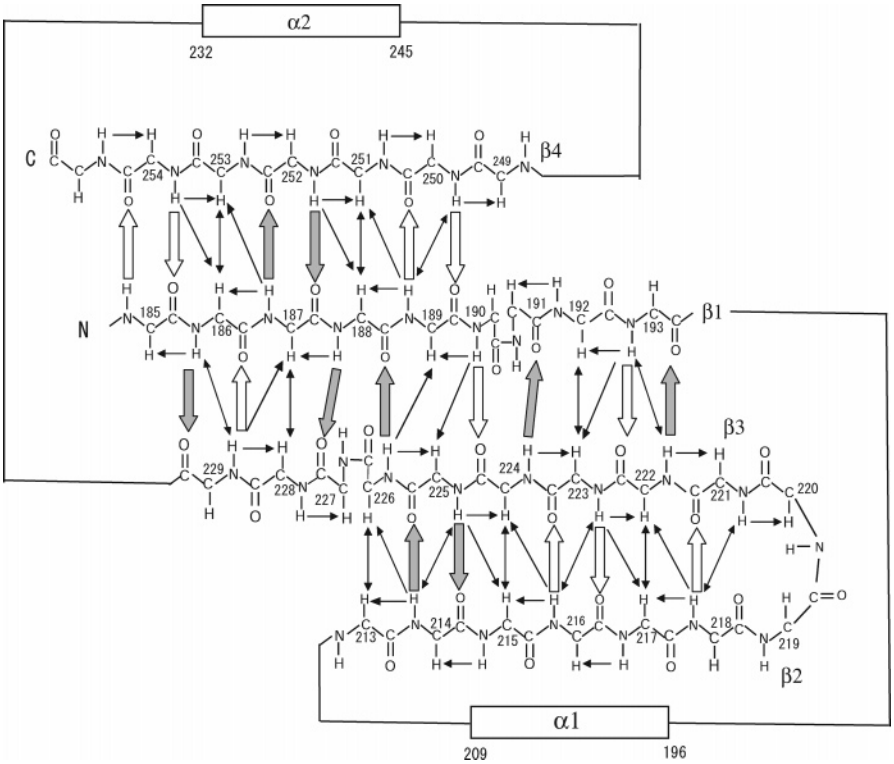

FIGURE 4: Schematic diagram of CwlCr secondary structure. The hydrogen bonds identified through scalar couplings across hydrogen bonds ($^{h3}J_{NC'}$) are displayed as wide arrows. Gray wide arrows represent hydrogen bonds observed in both CwlCr and CwlCr21 (see the text). The Y186 NH:F229 C′, K187 NH:L252 C′, V188 NH:G227 C′, I214 NH:Q225 C′, Y222 NH:F193 C′, V224 NH:G191 C′, I226 NH:V188 C′, Q225 NH:214 C′, and L252 NH:K187 C′ hydrogen bonds of CwlCr correspond to the Y3 NH:F46 C′, K4 NH:L69 C′, V5 NH:G44 C′, I31 NH:Q42 C′, Y39 NH:F10 C′, V41 NH:G8 C′, I43 NH:V5 C′, Q42 NH:I31 C′, and L69 NH:K 4C′ hydrogen bonds of CwCr21, respectively. The observed NOEs are represented with thin arrows. For simplicity, two α-helices are represented with white boxes.

Given the highly conserved repeated sequences within CwlCr, the chemical shifts of symmetrically positioned atoms were similar to each other, in particular, the two turn regions, I190, G191, A192, and F193 in repeat 1 and I226, G227, A228, and F229 in repeat 2 with identical amino acid sequence, where the Cα, Cβ, and C′ resonances of A192 and those of A228 were very similar to each other. Sequential assignment of these regions was clearly verified by the amide correlations obtained from the 3D (H)N(CO-TOCSY)-NH experiment, which provided the unambiguous sequential walk (Figure 3). Some peaks displayed longer-range connectivity, and these were valuable for establishing reliable assignments.

The side chain assignments were mainly obtained from the 3D C(CO)NH, H(CCO)NH, HCCH-TOCSY, and 4D HC(CO)NH spectra. Combined use of the 3D HCCH-TOCSY spectra with the 4D HC(CO)NH spectra enabled us to obtain unambiguous correlations between side chain ^{13}C and ^{1}H nuclei, which allowed for reliable side chain assignments. In an effort to obtain precise structural information, all of the methyl groups of leucine and valine were stereospecifically assigned using randomly 15% ^{13}C-enriched protein.

The structure of CwlCr was determined from 1206 distance and 134 torsion angle restraints (Table 1). This relatively large number of angle restraints was due to the φ and χ_1 angles obtained from TALOS, and the HNHB and HN(CO)-HB experiments, respectively. Furthermore, a total of 36 hydrogen bonds detected directly via $^{h3}J_{NC'}$ couplings were used as restraints (Figure 4). Of these, both the β-sheet and α-helices each possessed 18 hydrogen bonds. The direct observation of hydrogen bonds was extremely useful in the initial structure determination steps, since they facilitated the unambiguous determination of secondary structure topology, in particular, the β-sheet. Efficient NOESY cross-peak assignments were performed using CYANA version 1.05 with the CANDID protocol. Initially, 638 unambiguous NOEs were manually identified, and then the CANDID protocol provided approximately 500 restraints. Finally, the structure was calculated using CNS version 1.1, with 63 residual $^{1}D_{NH}$ dipolar couplings being added to the previously obtained distance and angle restraints.

Figure 5A depicts the backbone of the final 30 structures derived from the NMR data, showing that the atomic coordinates throughout the protein molecule have been well-defined except for the N- and C-terminal residues (residues 177−184 and 255, respectively). The average rms deviations calculated from the averaged structure were 0.16 and 0.52 Å for the backbone and all heavy atoms of the well-defined region (residues 185−254), respectively. The statistics of the structures are given in Table 1.

Structure of CwlCr. CwlCr adopts a $\beta\alpha\beta\beta\alpha\beta$-fold, comprising a layer consisting of an antiparallel β-sheet and two α-helices. The β-sheet is composed of β1 (residues 185−193), β2 (residues 214−218), β3 (residues 221−229), and β4 (residues 250−254) and is backed by α-helices α1 (residues 196−209) and α2 (residues 232−245) (Figures 1B and 5A,B). The main frame of the fold consists of a curled β-sheet, which resembles a horse saddle (Figure 5A,B).

The hydrogen bond network that defines the β-sheet topology is clearly identified by direct observation via $^{h3}J_{NC'}$

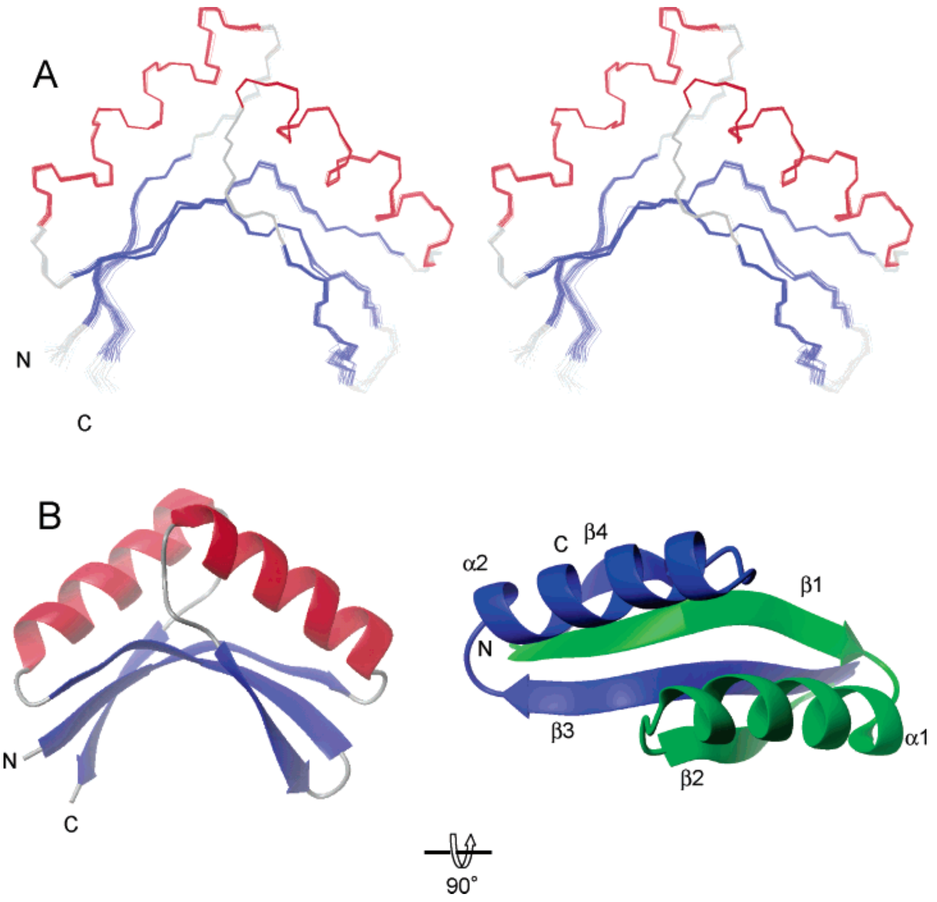

FIGURE 5: Solution structure of CwlCr. (A) Stereoview of the backbone superpositions of the final 30 simulated annealing structures of CwlCr. Helices and strands are colored red and blue, respectively. Residues 184−255 are shown. (B) Ribbon drawing of the representative structure of CwlCr in two different views. The molecular orientation in the left panel is the same as that in panel A, and the four-stranded β-sheet and α-helices are colored blue and red, respectively. The right panel shows a 90° rotation of the left panel about the horizontal axis. Repeat 1 and repeat 2 are colored green and blue, respectively.

couplings across the hydrogen bonds (Figure 4). The first strand, β1, forms a central part of the β-sheet with strand β3 in an antiparallel manner, and β4 and β2 contacted strands β1 and β3, respectively (Figures 4 and 5A,B). Additionally, $^{h3}J_{NC'}$ couplings show the presence of two β-bulges that are formed between strands β1 and β3 in the central part of the β-sheet. In the $^{h3}J_{NC'}$HNCO spectrum used for the detection of $^{h3}J_{NC'}$ couplings, a cross-peak was observed between the carbonyl group of G227 and the amide group of V188, while the amide group of I226 gave no detectable cross-peak. These observations indicated that a hydrogen bond is formed between G227 and V188, forming the β-bulge structure with a bend at the main chain position of G227 (Figure 4). Similarly, a cross-peak was observed between the carbonyl group of V191 and the amide group of V224, while the amide group of G190 gave no detectable cross-peak, indicating the presence of a hydrogen bond between V191 and V224, forming the β-bulge structure (Figure 4).

Interestingly, CwlCr is composed of two highly homologous repeat sequences that are 68% identical (Figure 1A). The first sequence repeat comprises (residues 184−219) strand β1, helix α2, and strand β2, while the second sequence repeat (residues 220−255) comprises strand β3, helix α2, and strand β4. Although CwlCr contains the two sequential sequence repeats, our structure determination revealed that the individual repeats do not form separate domains. Rather, the overall fold of the molecule is formed by numerous contacts between the two repeats (Figures 4 and Figure 5A,B). In addition to the aforementioned hydrogen bond network between the β-strands, the inward-facing residues on the β-sheet and α-helices form a hydrophobic core.

Conserved hydrophobic residues, noted as alignment positions *a*−*i* in Figure 1B, are likely to play key roles in the formation of the tertiary structure. Figure 6A shows the hydrophobic interactions within the protein core. I190 and I226 (position *c*) interact with each other, and are surrounded by V188 and V224 (position *b*) located at the center of the β-sheet (Figure 6A). L202 and L238 (position *f*) interact with F247 and F211 (position *h*), respectively, on the α-helices packing the core of the β-sheet (Figure 6A). A206 and A242 (position *g*) on the α-helices also form part of the core (Figure 6A).

Panels B and C of Figure 6 show the hydrophobic interactions on the edges of the protein. These stabilize the N-terminal tips of the α-helices on the β-sheet. A199 and A235 (position *e*) interact with V225 and V188 (position *b*), respectively, and also interact with V215 and V251 (position *i*), respectively, contributing to the interaction between the α-helices and the β-sheet. The aromatic rings of the conserved residues (positions *a* and *d*) interact with aliphatic β-methylene and the Hα atom. Y222 and Y186 (position *a*) interact with Hα atoms of K196 and K232, respectively. The Hα signals of these residues show significant upfield shifts (2.71 and 2.77 ppm, respectively) due to ring current effects. These observations confirm the interaction between the aromatic rings and the Hα atoms of the

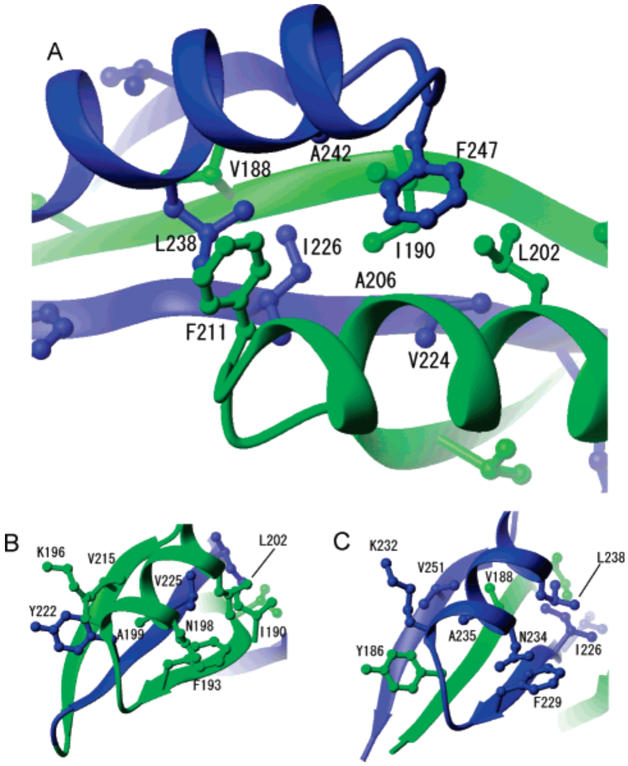

FIGURE 6: Hydrophobic interactions mediated by conserved residues. (A) Ribbon representation with a ball-and-stick model of the side chains at the protein core. (B and C) Hydrophobic interaction at the edges of the protein.

lysine residues. F193 and F229 (position *d*) interact with the β-methylenes of N198 and N234, respectively. The ^{1}H signals of these β-methylenes are well separated, implying rigidity of the side chain, and show upfield shifts due to ring current effects (chemical shifts of the upfield component of their signals are 1.93 and 1.98 ppm, respectively). This confirmed the interaction between the aromatic rings and the β-methylenes of the asparagines.

The residues (positions *a*−*i*) are completely conserved between repeat 1 and repeat 2 of CwlCr. They are located symmetrically within the structure, and are conserved throughout the sporulation repeat family according to the Pfam database (*16*), suggesting the existence of a common folding pattern composed of the two repeats similar to the CwlCr structure with numerous contacts between the two repeats. It should be noted that the sporulation repeats exist as two or more tandem repeats in most of the proteins that possess them. This supports the notion of a common fold consisting of two tandem repeat sequences. In fact, the C-terminal domain of FtsN consists of two tandem sporulation repeats folded into a similar structure showing a high *Z* score, 5.6, according to a DALI database search. The rms deviation between CwlCr and the C-terminal domain of FtsN (*40*) (PDB entry 1UTA) is 1.88 Å over 34 Cα coordinates for residues in the regions represented by secondary structure elements (CwlCr residues 187−192, 200−208, 212−215, 223−226, 238−245, and 250−252).

Circularly Permuted Repeat Mutant. Assuming that the core-forming conserved hydrophobic residues (*a*−*f*) are key determinants of the structural integrity, a circularly permuted repeat mutant may form a similar tertiary structure. In an effort to investigate this notion, we constructed a circularly permuted repeat mutant, termed CwlCr21(1−72) (Figure 1A). Recombinant CwlCr21 was expressed as a hexahistidine-tagged peptide in *E. coli* and subsequently purified by standard methods. No significant degradation had occurred during the purification process, and a relatively large amount of the recombinant protein was obtained (approximately 20 mg of ^{13}C- and ^{15}N-labeled CwlCr21/L of M9 culture). This was in contrast to the expression and purification of each single repeat being associated with a low yield due to degradation (data not shown). These results implied that CwlCr21 would also form a stable structure by the two repeats.

Furthermore, the ^{1}H−^{15}N HSQC spectrum of CwlCr21 shows well-dispersed and relatively sharp signals, indicating that CwlCr21 adopts a well-packed structure (Figure 7A). The main chain resonance assignments were obtained from HNCACB, HN(CO)CACB, HN(CA)CO, and HNCO experiments, and confirmed by HN(CO-TOCSY)NH experiments. The chemical shift index (*41*) of Cα and the chemical shift deviations of Cα and Cβ (represented as δCα − δCβ) (*42*) indicate that the secondary structure of CwlCr21 consists of $\beta\alpha\beta\beta\alpha\beta$, which is almost identical to that of CwlCr (Figure 7A). Additionally, the $^{h3}J_{NC'}$HNCO experiment shows the presence of hydrogen bonds between the β-strands (Figure 4). The observed hydrogen bonds indicate that strands β1−β4 form a single antiparallel β-sheet layer. Although the observed hydrogen bonds can only partially be determined given the quality of the spectrum, enough information has been gleaned to determine that this β-sheet topology is identical to that of CwlCr. (The strips of the $^{h3}J_{NC'}$HNCO spectra recorded on CwlCr and CwlCr21 are shown in Figures S1 and S2 of the Supporting Information.)

These data imply that the tertiary structure of CwlCr21 is similar to that of CwlCr. Therefore, the repeat sequences are exchangeable, which confirms that the conserved core-forming residues are key determinants of protein folding. This reinforces the notion of a common folding pattern formed by two sporulation-related repeats.

Domain-Swapped Dimer? The CwlCr21 data indicate that the repeat sequences of CwlCr are exchangeable. This result raises another concern that CwlCr forms a domain-swapped dimer. Nevertheless, fine agreement exists between the observed residual dipolar couplings and the determined structure (Table 1), indicating that almost all of CwlCr exists as a monomer in solution under normal conditions. Furthermore, analytical gel-filtration chromatography using Superdex-75 linked to an AKTA Purifier system (Amasharm Biosciences) showed that CwlCr exits as a monomer, with no peaks corresponding to dimer (or higher multimer) molecular weights being detected (data not shown). Thus, we conclude that CwlCr exists as a monomer under normal conditions.

To date, several domain-swapped dimers have been reported (*43*−*45*). The domain-swapped dimers are in a meta-stable, kinetically trapped state at room temperature. Thus, in formation of a domain-swapped dimer, it is necessary to ensure transient destabilizing conditions such as low pH, and the presence of organic solvents or chaotropic agents at high protein concentrations (*43*, *45*). These destabilizing conditions were not tested in this study. The generation of a CwlCr domain-swapped dimer remains an interesting objective from a protein engineering viewpoint.

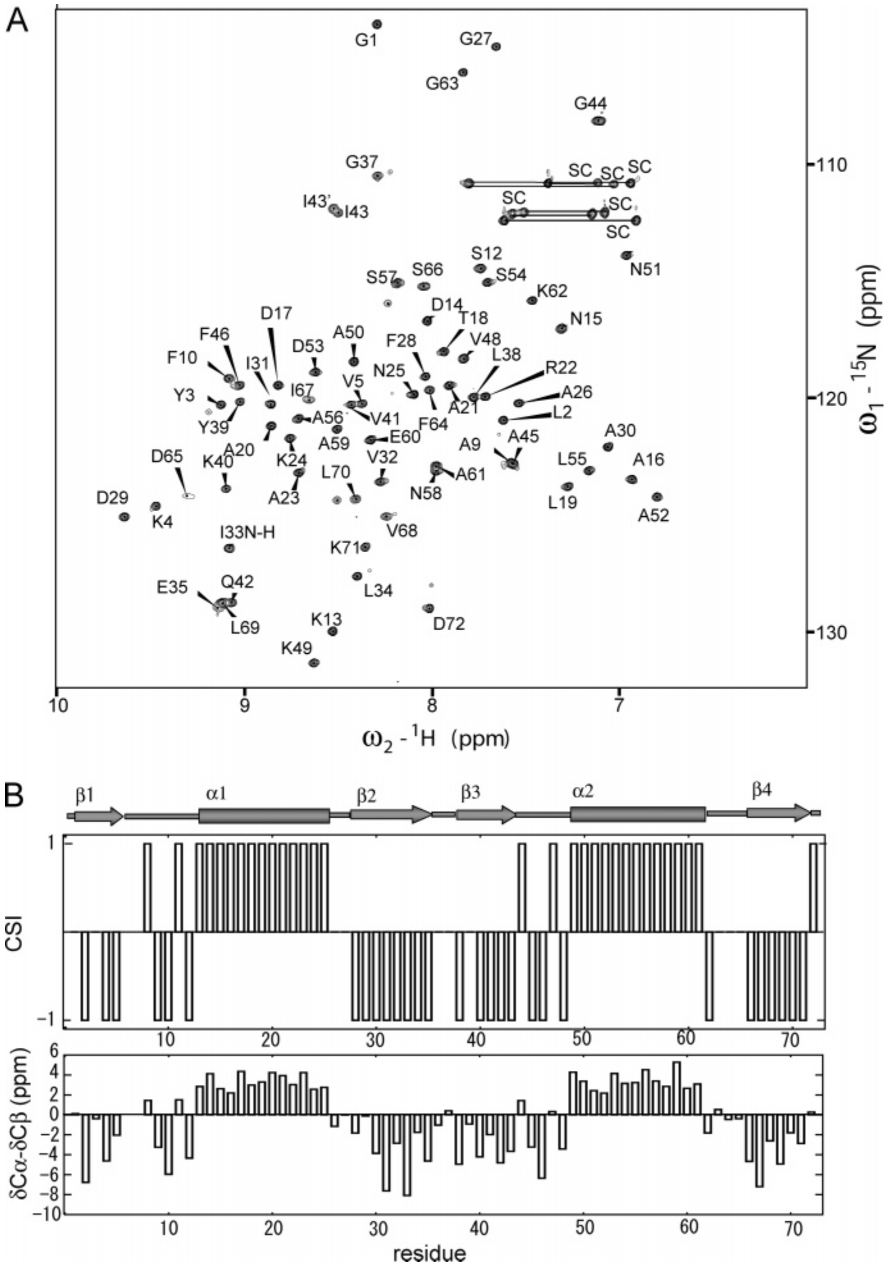

FIGURE 7: (A) ^{15}N$-$^{1}H HSQC spectrum of CwlCr21. The spectrum was obtained with 0.8 mM CwlCr21 at pH 6.5 and 30 °C on a Bruker DRX 800. The SC label indicates side chain peaks of asparagine or glutamine residues. (B) Secondary structure of CwlCr21. The top panel shows the score of the chemical shift index of the Cα atom. Estimated secondary structure is shown at the top of the panel. The bottom panel shows chemical shift deviations of Cα and Cβ atoms. Deviations from the random values of Cα and Cβ are shown as δCα $-$ δCβ.

Peptidoglycan Binding. Titration experiments were employed using ^{1}H$-$^{15}N HSQC spectra in an effort to establish the peptidoglycan interaction surfaces of CwlCr. However, since peptidoglycan obtained from *B. subtilis* was insoluble in aqueous solution, NMR experiments could not be applied in a straightforward manner.

Thus, we initially tried to monitor the interactions between CwlCr and the building blocks of the peptidoglycan, (1) diaminopimelic acid, (2) Ala-D-γ-Glu-Lys-D-Ala-D-Ala, (3) GlcNAc, and (4) GlcNAcMurNAcAlaGln, since these molecules could be readily dissolved in aqueous solution. We expected to obtain some clues pertaining to the binding sites, even if the binding was predicted to be rather weak compared to that expected for intact peptidoglycan. However, addition of a large excess of these molecules to CwlCr showed no signal perturbations, suggesting that these building blocks in and of themselves were insufficient for CwlCr binding, or that the binding was too weak to be detected by NMR (data not shown).

We next improved the solubility of the peptidoglycan by enzymatic digestion with intact CwlC. The digested short peptidogylcan fragment was soluble up to 0.5 mg/mL. Figure 8A shows the signal perturbation following the addition of digested products. To identify the peptidoglycan binding regions of CwlCr, significantly affected residues were mapped on the structure of CwlCr. The residues are essentially confined to two regions, the N-terminal tip of the two α-helices and the β-sheet regions near these on the edges of the protein (sites 1 and 2) (Figure 8B). The exposed residues are K194, V195, K196, A197, N198, D200, and S201 in site 1 and S230, S231, K232, D233, N234, D236, and T237 in site 2. In particular, N198 and N234, which are

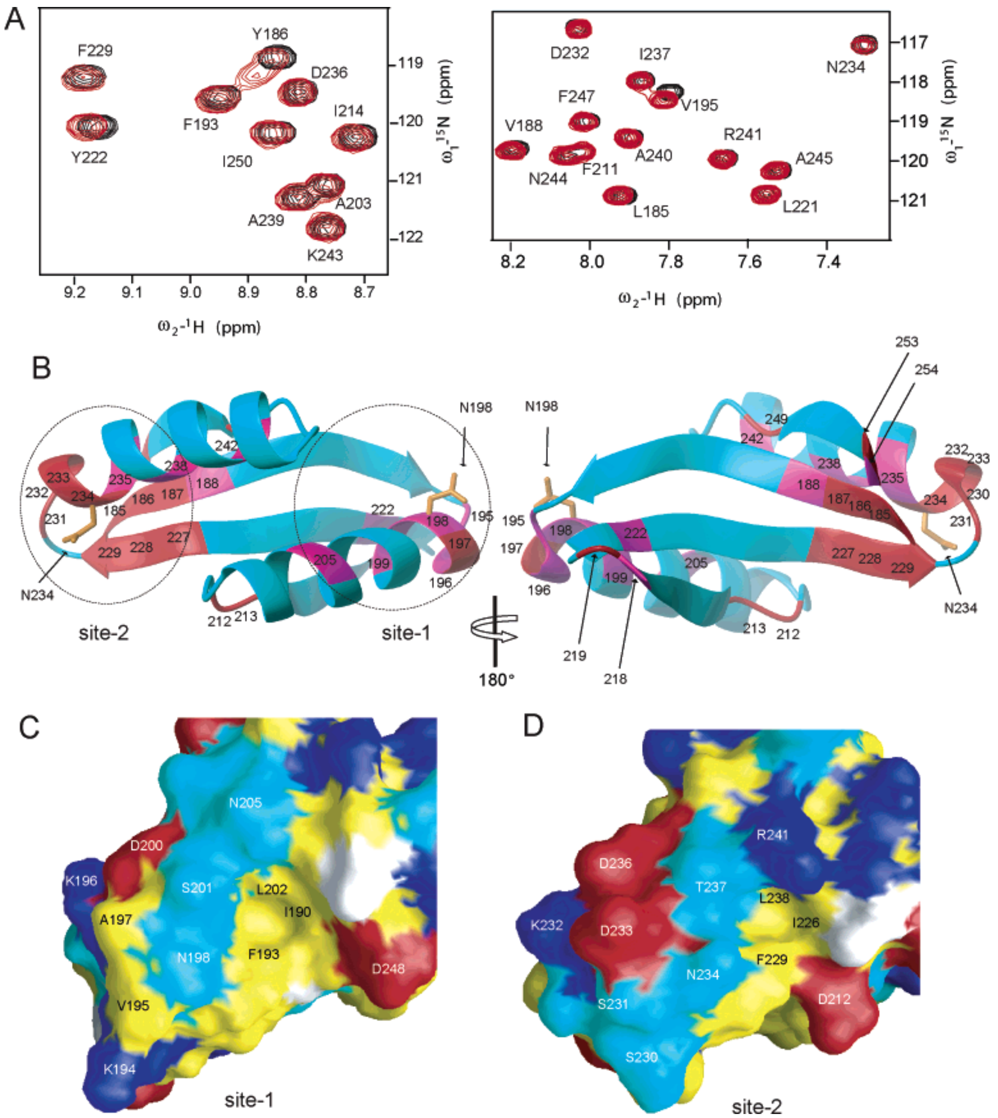

FIGURE 8: Chemical shift perturbation experiments. (A) Selected region of the ^{15}N$-$^{1}H HSQC spectra of CwlCr, recorded in the absence (black) or presence (red) of 0.5 mg/mL digested peptidoglycan. The threshold of the perturbed spectrum is set to 20-fold lower than that of the control for clarity. (B) Representation of the ribbon model with color coding to show the effect following the addition of 0.5 mg/mL digested peptidoglycan. Residues that show weighted average shift difference values, $\delta_{ave}/\delta_{max}$, larger than 0.25 are colored pink with the residue number ($\delta_{max} = 0.046$ ppm). Residues whose signal intensity [$(I_{ref} - I_{per})/I_{ref}$] is reduced to >96% of the original values are colored red with the residue number. Residues whose value of both weighted average shift difference and intensity reduction are larger than the threshold are also colored red. Side chains of Asn198 and Asn234 are shown as orange sticks. Patches with confined perturbed residues are circled. (C and D) The molecular surfaces of sites 1 and 2 of CwlCr are shown. The molecular orientations in panels C and D are the same as in panels B and C of Figure 6, respectively. The figures were prepared using GRASP (*56*).

exposed on the surface at the center of the two perturbed sites, represent conserved invariant residues as found with other cell wall lytic enzymes (Figures 1B and 8C,D).

Mutation analyses showed that the N198D and N234D double mutation completely impaired peptidoglycan binding, while each single mutant retained ~75% binding activity, indicating that these two residues participate in peptidoglycan binding. Extensive site-directed mutagenesis and the significance of the findings to binding activity will be published elsewhere (T. Shida et al., manuscript in preparation). N198 and N234 residues are exposed on the edges of the domain, the equivalent positions on the symmetrical structure, and are located approximately 28 Å from each other (Figure 8B). The local environments around N198 and N234 are also equivalent, and involve contact with the conserved aromatic ring of phenylalanines, F193 and F229, respectively (position *d*) (Figure 6B,C). These observations suggest that CwlCr possesses two equivalent symmetrically located binding sites. The chemical shift perturbations of site 2 were more affected than those of site 1, but this may not necessarily reflect a difference in the peptidoglycan binding preference of the two sites. Although both site 1 and site 2 are composed of the tip of the α-helix and β-sheet, the β-sheet of site 2 includes the N-terminal and C-terminal region, in contrast to that of site 1 which includes the β-hairpin (Figures 6B and 8B). Thus, NMR signals of site 2 are likely to be more susceptible to peptidoglycan binding.

Surface properties of sites 1 and 2 are shown in Figure 8C,D. In both sites, concave hydrophobic patches exist near the key asparagine residues, N198 and N234. Portions of the side chains of I190, F193, and L202 form the patch in site 1, and those of I226, F229, and L238 form the patch in site 2 (Figure 8C,D). These hydrophobic residues are highly conserved. This suggests the conserved hydrophobic surfaces adjacent to the polar side chains of the asparagines are important for CwlCr−peptidoglycan interaction. In contrast, functional groups of the side chains of K194, V195, and A197 in site 1 and S230, S231, and D233 in site 2, which are located at the turns connecting β1 and α1, and β3 and α2, and are located at tips of the helices, are not conserved between the two sites (Figure 8C,D) or among other cell wall lytic enzymes, implying these residues are not important for peptidoglycan binding. Chemical shift perturbations of these residues are likely to reflect conformational changes of the turns caused by binding of peptidoglycan to the flanking polar−hydrophobic regions but not direct binding.

The peptidoglycan layer consists of a carbohydrate backbone of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid. The *N*-acetylmuramic acid residues are cross-linked with the oligopeptide linker, including a branch formed by diaminopimelic acid (*46*, *47*). The layers stacked, and carbohydrate backbones and oligopeptide linkers form lattice-like three-dimensional structures. Thus, bivalent binding exerted by two equivalent symmetrical binding sites on pseudo-2-fold symmetric structure may be advantageous, since it increases the affinity for peptidoglycan consisting of quasi-periodic structures.

By analogy, it was reported that Cyanovirin-N binds N-linked high-mannose oligosaccharides on gp120 through two major sugar binding sites (*48*). Besides, in the case of lectin−oligosaccharide binding, many naturally occurring oligosaccharides and glycoconjugates are reported to be multivalent, thereby increasing the apparent affinity for lectins relative to monovalent analogues, implying that multivalent binding is advantageous (*49*).

Some tertiary structures typical of a “peptidoglycan binding domain as a protein module” have been reported, including the LysM domain (*50*) and PGRPs (*51*−*53*). In the case of LysM and PGRP, however, multivalent binding was not reported. The difference in the binding mode may reflect the difference in their tertiary structure. That is, CwlCr and Cyanovirin-N adopt a 2-fold symmetric structure, unlike LysM and PGRP.

Obviously, further quantitative biochemical analyses of CwlCr and peptidoglycan interactions, such as the number of binding sites and stoichiometry, are required. At present, more detailed analyses have been hampered by difficulties in handling peptidoglycan. Access to and use of a new variety of chemically synthetic peptidoglycan building blocks may allow us to overcome the inherent problems associated with the use of peptidoglycan.

Conclusion. We have determined the tertiary structure of the sporulation-related repeats in CwlC amidase, CwlCr. Because of the characteristic repeat sequences, the ^{1}H and ^{13}C resonances heavily overlapped, and thus, advanced NMR techniques were required to determine the structure, such as 3D (H)N(CO-TOCSY)-NH for the assignments, $^{h3}J_{NC'}$HNCO for the hydrogen bond constraints, and ^{1}H−^{15}N IPAP for the residual $^1D_{NH}$ couplings. The repeat sequence adopts a remarkable 2-fold symmetric structure, consisting of a $\beta\alpha\beta\beta\alpha\beta$-fold. The repeats of CwlCr exhibit interesting behavior in that the repeats can be swapped, and the circularly permuted variant forms a similar tertiary structure, indicating that the conserved hydrophobic residues are keys for folding of the repeats. Chemical shift perturbation experiments indicate that CwlCr directly binds to peptidoglycan, and at least two interaction sites on the edges of the protein have been identified.

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SUPPORTING INFORMATION AVAILABLE

Strip plot of the 3D $^{h3}J_{NC'}$ spectrum of CwlCr (Figure S1) and CwlCr21 (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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