Charakterisierung des Transkriptionsfaktors CcpN aus

*Bacillus subtilis*

Dissertation
zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät
der Friedrich-Schiller-Universität Jena

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Datum der Disputation: 26.11.2009
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1. Einleitung

1.1. Transkriptionsregulation in Prokaryoten

1.1.1. Die Transkriptionsinitiation

Bakterien sind im Laufe ihres Daseins mit einer Vielzahl unterschiedlicher Umweltbedingungen konfrontiert, auf welche sie angemessen reagieren müssen. Während geringe Schwankungen z. B. der Osmolarität oder der Konzentration intrazellulärer Metabolite durch die Aktivität entsprechender Porine oder Stoffwechselenzyme ausgeglichen werden können, bedürfen größere und langfristig anhaltende Schwankungen einer intensiveren Regulation. Da auf sich ändernde Umweltbedingungen in der Regel mit einer Änderung der Protein zusammensetzung der Zelle reagiert werden muss, steht Bakterien hier nur eine begrenzte Anzahl an Mechanismen zur Verfügung. Es kann zum einen die Menge an Protein reguliert werden, entweder durch Kontrolle der Syntheserate oder durch Kontrolle des Abbau s, zum anderen kann die Menge an mRNA für das entsprechende Protein reguliert werden. Letzteres ist wiederum entweder durch Regulation der Synthese oder des Abbau s möglich, wobei ersteres die deutlich ökonomischere Variante darstellt, da bereits die Synthese einer mRNA einen Energieaufwand für die Zelle bedeutet.

Die Regulation der Synthese einer RNA findet in der Regel während der Transkriptionsinitiation, seltener während der Elongation statt. Die Initiation der Transkription ist ein komplexer Prozess, der aus einer sequentiellen Abfolge mehrerer Schritte besteht (Record et al., 1996; Abbildung 1). Im ersten Schritt bindet die bakterielle RNA-Polymerase (RNAP), ein aus mehreren Untereinheiten bestehendes Enzym, an eine Promotor-Region. Die Polymerase selbst besteht aus einer $\beta$- und einer $\beta'$-Untereinheit, die das katalytische Zentrum bilden (Korzheva et al., 2000). Die Assemblierung dieser beiden Untereinheiten wird durch die N-terminale Domäne der $\alpha$-Untereinheiten ($\alpha$-NTD) unterstützt (Blatter et al., 1994), die C-terminale Domäne ($\alpha$-CTD) besitzt unterstützende Funktion bei der Promotorerkennung und -bindung (Gourse et al., 2000). Der $\sigma$-Faktor schließlich ist für die Erkennung der Promotor-Sequenz und die Rekrutierung der RNAP an den Promotor verantwortlich (Wöst en, 1998). Neben diesen notwendigen Untereinheiten gibt es mehrere, die mit der RNAP assoziiert sein können, ihre Funktion unterstützen, aber für eine Promotorerkennung und Transkription nicht zwingend notwendig sind. Beispiele hierfür sind die $\omega$-Untereinheit, die als Chaperon die korrekte Faltung der $\beta'$-Untereinheit unterstützt.
Einleitung


Abbildung 1:
Phasen der Transkriptionsinitiation

Die verschiedenen Komplexe, die im Zuge des Transkriptionsinitiationsprozesses gebildet werden, sind schematisch dargestellt. Bis auf den Übergang in den Elongationskomplex sind alle Schritte reversibel.

Die Regulation des Transkriptionsinitiationsprozesses kann nun nicht nur an jedem dieser Schritte ansetzen, sondern auch in jede beliebige Richtung erfolgen: Proteine, die die Aktivierungsgenergie eines bestimmten Schrittes senken, wirken als Aktivatoren, solche, die die Aktivierungsgenergie erhöhen, wirken als Repressoren.

1.1.2. Regulation durch Promotor-Selektivität

Transkriptionsregulation kann auf einer basalen Ebene durch die Sequenz eines Promotors stattfinden. Promotoren, deren Elemente nahe an der Konsensus-Sequenz sind, sind in der Regel effizienter als solche mit stark abweichenden Sequenzen. Da diese Regulation allerdings statisch in der DNA-Sequenz verankert ist, dient sie lediglich dazu, die RNAP ungleichmäßig auf verschiedene Promotoren zu verteilen und so eine generelle Kontrolle der Transkriptmenge zu erreichen.

Einen Spezialfall unter den Regulatoren der Transkription stellen die σ-Faktoren dar. Verschiedene Organismen besitzen eine unterschiedliche Menge an σ-Faktoren, von einem einzigen bei Mycoplasma genitalium bis hin zu 63 bei Streptomyces coelicolor (Gruber & Gross, 2003). Jeder σ-Faktor besitzt unterschiedliche Voraussetzungen bezüglich der Sequenz der −10- und −35-Region (die oben angegebenen Konsensussequenzen gelten für den Haupt-σ-Faktor), wodurch die RNAP durch verschiedene σ-Faktoren gezielt zu einem bestimmten Satz an Promotoren geführt werden kann. Eine Regulation der σ-Faktor-Aktivität kann zum einen durch dessen Synthese und Abbau stattfinden, zum anderen durch sogenannte Anti-σ-
Faktoren. Diese Proteine binden einen spezifischen σ-Faktor und verhindern so, dass dieser die RNA-Polymerase binden kann (Hughes & Mathee, 1998).

1.1.3. Regulation durch Transkriptionfaktoren

1.1.3.1. Aktivierung


Abbildung 2:
Mechanismen der Transkriptionsaktivierung
Aktivatoren sind mit einem A gekennzeichnet und als Dimer dargestellt, da sie oft als Dimer arbeiten, RNAP stellt die RNA-Polymerase dar, α die α-Untereinheit, σ den σ-Faktor.
(a): Klasse-I-Aktivierung. Der Aktivator bindet upstream des Promotors und rekrutiert die RNA-Polymerase durch Kontakte zu den α-CTDs.
Einleitung


Einen Sonderfall bilden Promotoren, die von σ^{54} von *E. coli* erkannt werden. Diese sind in der Regel durch die geringe Stabilität des offenen Komplexes limitiert und benötigen Aktivatoren, die spezifisch den offenen Komplex stabilisieren (Buck *et al.*, 2000).

1.1.3.2. Repression


Abbildung 3:
Mechanismen der Transkriptionsrepression
Repressoren sind mit einem R gekennzeichnet und als Dimer dargestellt, da sie oft als Dimer arbeiten, RNAP stellt die RNA-Polymerase dar, α die α-Untereinheit, σ den σ-Faktor
(a): Inhibierung der RNAP-Bindung. Durch Bindung an die Promotorregion blockiert der Repressor die Bindungsstelle für die RNA-Polymerase.
(b): Inhibierung der Bildung des offenen Komplexes. Trotz Überlappung der Bindungsstellen können RNAP und Repressor gleichzeitig binden, jedoch erlaubt die Anwesenheit des Repressors keine Bildung eines stabilen offenen Komplexes.
(c): Inhibierung der Transkriptionsinitiation. Der Repressor und die RNAP können gleichzeitig binden und es wird auch ein stabiler offener Komplex gebildet. Die Anwesenheit des Repressors inhibiert allerdings jegliche Transkription, es werden weder abortive noch komplette Transkripte gebildet.
(d): Inhibierung der Bildung eines Elongationskomplexes. Der Repressor arretiert die RNAP am Promotor, entweder durch direkte Interaktion mit einer RNAP-Untereinheit (oben) oder durch Bildung einer Blockade downstream der RNAP (unten). Abortive Transkripte werden gebildet, die RNAP kann den Promotor jedoch nicht verlassen.
An zahlreichen Promotoren wird der Übergang vom geschlossenen zum offenen Komplex durch Repressoren beeinflusst. Diese Repressoren können Operatoren besetzen, die direkt mit dem Promotor überlappen, jedoch trotzdem die gleichzeitige Bindung von Repressor und RNAP erlauben, wie zum Beispiel der MerR-Repressor am merT-Promotor, für den in vitro und in vivo eine Inhibierung der Bildung des offenen Komplexes gezeigt wurde (Heltzel et al., 1990), oder das Spo0A-Protein aus B. subtilis am abrB-Promotor (Greene & Spiegelman, 1996). Es existieren allerdings auch Repressoren der Bildung des offenen Komplexes, deren Operatoren nicht mit der RNAP-Bindungsstelle überlappen, wie der Repressor KorB am korABF-Promotor (Williams et al., 1993). Einen Spezialfall stellen hier wieder σ54-kontrollierte Promotoren dar, bei denen, wie oben beschrieben, die Bildung des offenen Komplexes durch Aktivatoren gewährleistet werden muss. An diesen Promotoren existiert ein Repressionsmechanismus, bei dem ein Repressor zwischen Aktivator und RNAP bindet, dadurch eine DNA-Biegung induziert, was letztendlich die Interaktion zwischen Aktivator und RNAP verhindert. Als Beispiele hierfür seien der Nac-Regulator aus Klebsiella aerogenes (Feng et al., 1995) oder auch der globale Regulator CcpA aus B. subtilis bei der Regulation des lev-Operons genannt (Martin-Vestraete et al., 1995).

Für eine Repression des folgenden Schrittes, der Bildung des Initiationskomplexes und der damit einhergehenden abortiven Transkripte, gibt es nur wenige Belege in der Literatur. Ein derartiger Mechanismus wurde für das H-NS-Protein am rrnB P1-Promotor (Schröder & Wagner, 2000) und das FIS-Protein am gyrB-Promotor berichtet (Schneider et al., 1999). In beiden Fällen wurde gezeigt, dass offene Komplexe gebildet werden, jedoch waren keinerlei Transkripte nachweisbar.


1.1.4. Regulation der Transkriptionsregulatoren


1.2. Katabolitrepression in B. subtilis

Der Metabolismus der meisten Bakterien ist dazu ausgelegt, eine Vielzahl von möglichen Nahrungsquellen zu verwerten. Allerdings sind für die Erschließung vieler dieser

1.2.1. Elemente des CCR-Systems in B. subtilis

Fujita, 1990), wobei festgestellt wurde, dass die meisten der natürlich vorkommenden cre-Sequenzen geringe Abweichungen vom Konsensus aufweisen und diese Abweichungen für ihre jeweilige Funktion notwendig sind (Miwa et al., 2000). Da der an die cre-Sequenz bindende Transkriptionsfaktor CcpA konstitutiv exprimiert wird (Miwa et al., 1994), wurde schnell klar, dass es noch mindestens einen weiteren Faktor geben muss, der in die Katabolitrepression bei B. subtilis involviert ist.


1.2.2. CcpA-abhängige Katabolitrepression

Eine Phosphorylierung von HPr am Ser46 hat dagegen eine völlig andere Funktion. Die Phosphorylierung an dieser Position wird durch die HPr-Kinase/Phosphatase katalysiert, die wiederum von steigenden Konzentrationen Fructose-1,6-Bisphosphat aktiviert wird, also immer dann, wenn ausreichend Nährstoffe und damit Glycolyseintermediate vorhanden sind (Reizer et al., 1998). In diesem Phosphorylierungszustand (HPr-Ser46-P) ist HPr in der Lage, mit dem Transkriptionsfaktor CcpA zu interagieren und dessen Fähigkeit, cre-Elemente zu binden, signifikant zu erhöhen (Deutscher et al., 1995; Jones et al., 1997). Die Phosphorylierung am Ser46 ist reversibel und kann durch die Phosphatase-Aktivität der HPr-Kinase/Phosphatase entfernt werden. Die Phosphatase-Aktivität wird durch Phosphat-Ionen stimuliert, die auf einen niedrigen intrazellulären ATP-Spiegel und dadurch schlechte metabolische Bedingungen hindeuten (Hanson et al., 2002). Dies erklärt auch, wie das
Einleitung

(a) Abwesenheit von Glucose

1: In der frühen Phase der Glucoseverwertung wird HPr an Ser46 phosphoryliert und kann dadurch mit CcpA Heterodimere bilden. HPr-Ser46-P kann nicht mehr am His15 phosphoryliert werden.

2: In der späten Phase der Glucoseverwertung wird HPr an Ser46 phosphoryliert und kann dadurch mit CcpA Heterodimere bilden. CcpA kann mit HPr-Ser46-P heterodimerisieren und die Transkription an Promotoren katabolischer Operons, die cre-Elemente enthalten, regulieren.

(b) Anwesenheit von Glucose

Abbildung 4:
Vereinfachte Übersicht über das Katabolitpressionssystem in *B. subtilis*
Die Abbildungsbeschreibung befindet sich auf der nächsten Seite.
Einleitung


Cre-Elemente, die mit der Promotor-Region überlappen, vermitteln in den meisten Fällen durch eine sterische Behinderung der RNAP-Bindung Katabolitrepression, wie z. B. bei den amyE-, bglP- und dctP-Genen, welche verschiedene Abbauenzyme oder Transporter für alternative Nahrungsquellen wie Stärke, Disaccharide oder diverse C₄-Dicarboxylate codieren (Nicholson et al., 1987; Krüger et al., 1996; Asai et al., 2000). Ein häufig beobachteter

Abbildung 4:
Vereinfachte Übersicht über das Katabolitrepressionssystem in B. subtilis


(1). Wenn sich Glycolyseintermediate wie Fructose-1,6-Bisphosphat anreichen, wird die Kinase-Aktivität der HPr-Kinase/Phosphatase stimuliert und HPr am Ser46 phosphoryliert, wonach es nicht mehr für die Phosphorylierung der Glucose zur Verfügung steht. HPr-Ser46-P kann nun seinerseits mit CcpA heterodimerisieren und die Transkription an Promotoren mit cre-Elementen regulieren.

(2). Sinkt die ATP-Konzentration in der Zelle, was mit einer steigenden Phosphat-Konzentration einhergeht, wird die Phosphatase-Aktivität der HPr-Kinase/Phosphatase aktiviert. Dadurch kann HPr nicht mehr mit CcpA interagieren und dieses die cre-Elemente nicht mehr binden. Die Phosphorylierungszustände His15-P und Ser46-P schließen sich gegenseitig aus.


Durch bioinformatische Analyse des B. subtilis-Genoms wurde ein Protein entdeckt, das eine dem HPr ähnliche Sequenz aufweist: Crh (Galinier et al., 1997). Es wurde gezeigt, dass dieses Protein durch die HPr-Kinase/Phosphatase phosphoryliert werden kann und auch mit CcpA interagiert. Eine genaue Funktion dieses Proteins ist noch nicht bekannt, es gibt jedoch Hinweise darauf, dass es die CCR-Funktion von HPr beim Wachstum auf Nicht-
Kohlenhydrat-Substraten wie Succinat oder Glutamat übernimmt (Warner & Lolkema 2003, Görke et al., 2004).

1.2.3. CcpA-unabhängige Katabolitrepression

Obwohl CcpA einen Großteil der unter Katabolitrepression und -aktivierung stehenden Gene der Zelle kontrolliert, zeigten Micro-Array-Analysen, dass es einige Gene gibt, die auch in einem CcpA-Knockout-Stamm oder einem Stamm, in dem HPr nicht an Ser46 phosphoryliert werden kann, durch die Anwesenheit von Glucose im Medium reprimiert werden (Yoshida et al., 2001; Lulko et al., 2007). Dies führte zur Identifizierung weiterer Katabolit-Kontroll-Proteine, die im Folgenden kurz vorgestellt werden sollen:

CcpB, ein zu CcpA paraloges Protein, ist an der Katabolitrepression einiger weniger katabolischer Operons zur Verwertung von Gluconat und Xylose beteiligt und scheint vor allem beim Wachstum auf festen Medien aktiv zu sein, wurde aber seit seiner Entdeckung vor über 10 Jahren nicht weiter erforscht (Chauvaux et al., 1998).

CcpC reprimiert die Gene citZ, citB und citC, die die Enzyme der ersten drei Schritte des Citrat-Zyklus codieren (Jourlin-Castelli et al., 2000). Dabei wird CcpC zum einen durch CcpA und zum anderen durch sich selbst negativ reguliert (Kim et al., 2002; Kim et al., 2003), was die Repressorkonzentration auf ein niedriges, aber für effiziente Repression ausreichendes Niveau reduziert. Citrat wirkt als negativer Regulator von CcpC, sodass der Citrat-Zyklus wieder ablaufen kann, sollte sich Citrat in größeren Mengen in der Zelle ansammeln. Dies und der Wegfall der Repression durch CcpA führen außerdem zu einer Erhöhung der Konzentration an CcpC, sodass die Repression des Citratzyklus bei plötzlicher Citratknappheit sofort wieder einsetzen kann.

Einleitung


1.3. Der Transkriptionsfaktor CcpN

Der letzte bisher entdeckte Transkriptionsfaktor, der an der Katabolitrepression beteiligt ist, wurde CcpN (catabolite control protein of gluconeogenic genes) genannt, da er im Zuge der Aufklärung der Regulation der beiden Gene pckA (PEP-Carboxykinase) und gapB (Glycerinaldehyd-3-Phosphat-Dehydrogenase B), die ausschließlich in der Gluconeogenese aktiv sind, entdeckt wurde (Servant et al., 2005). Zeitgleich dazu wurde CcpN im Rahmen meiner Diplomarbeit unabhängig davon als Transkriptionsregulator einer kleinen RNA, SR1, identifiziert (Licht et al., 2005). SR1 ist ein negativer Regulator von AhrC, das wiederum Gene des Arginin-Abbaus positiv und der Arginin-Biosynthesegene negativ reguliert (Heidrich et al., 2006). Es konnte gezeigt werden, dass SR1 durch Basenpaarung mit der ahrC-mRNA deren Struktur ändert und dadurch die Translationsinitiation inhibiert (Heidrich et al., 2007). Für alle drei Gene, sr1, pckA und gapB, konnte eine starke Repression in Anwesenheit einer beliebigen in die Glycolyse einfließenden Zuckerquelle, wie z. B. Glucose, Fructose oder Glycerin gezeigt werden. Servant et al. zeigten, dass CcpN für effizientes Wachstum unter glycolytischen Bedingungen essentiell ist und ccpN-Knockout-Stämme stark in ihrer Wachstumsgeschwindigkeit limitiert sind. Unter gluconeogenetischen Bedingungen konnte ein schwacher wachstumsbeschleunigender Effekt eines ccpN-Knockout-Stammes nachgewiesen werden (Servant et al., 2005).

bei der Bindung an die DNA mehrere DNase-I-hypersensitive Stellen erzeugt, was in der Regel auf eine Änderung der DNA-Struktur hinweist (Servant et al., 2005; Licht et al., 2005). Licht et al. konnten jedoch anhand des sr1-Promoters zeigen, dass ein Operator ausreichend ist, im CcpN binden zu können. Mit Hilfe von EMSAs mit mutierten Oligonucleotiden konnte folgende provisorische Konsensussequenz für CcpN abgeleitet werden: DDDTGTTGYYATACTRDK. Eine Suche nach dieser Sequenz im B. subtilis-Genom zeigte, dass zahlreiche Gene einen CcpN-Operator in der Promotorregion besaßen, darunter auch pckA und gapB. Allerdings wiesen nur diese beiden neben dem sr1-Operator Bindungsaktivität auf.


1.4. Zielsetzung


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Bereits bei der ersten Charakterisierung von CcpN gab es Hinweise, dass die intrazelluläre Konzentration dieses Repressors verhältnismäßig hoch sein muss. Im dies zu verifizieren, sollte die Konzentration von CcpN in der Zelle bestimmt werden. Im Anschluss daran sollte mit Hilfe von bioinformatischen und molekularbiologischen Mitteln nach weiteren CcpN-
regulierten Genen im Genom von *B. subtilis* gesucht werden. Dazu sollten potentielle CcpN-Operatoren in EMSAs auf ihre Proteinbindungsfähigkeit untersucht und anschließend mittels DNase I- und Interferenz-Footprints genauer untersucht werden, um Gemeinsamkeiten mit und Unterschiede zu den bereits bekannten Promotoren festzustellen. Positive Kandidaten sollten anschließend mit Hilfe von *lacZ*-Transkriptionsfusionen auf ihre Funktionalität *in vivo* untersucht und die Ergebnisse bei Bedarf durch *in vitro*-Transkriptions-Versuche untermauert werden (*Manuskript IV*).
2. Übersicht zu den Manuskripten

**Manuskript I**
Andreas Licht & Sabine Brantl  
*Transcriptional repressor CcpN from *Bacillus subtilis* compensates asymmetric contact distribution by cooperative binding.*  


**Manuskript II**
Andreas Licht, Ralph Golbik & Sabine Brantl  
*Identification of ligands affecting the activity of the transcriptional repressor CcpN from *Bacillus subtilis*.  


Alle Experimente in dieser Publikation wurden von Andreas Licht erdacht und durchgeführt. Ralph Golbik führte die Reinigung von CcpN mittels FPLC durch, vermittelte

Manuskript III
Andreas Licht & Sabine Brantl

The transcriptional repressor CcpN from *Bacillus subtilis* uses different repression mechanisms at different promoters.

Revision eingereicht bei *Journal of Biological Chemistry* am 03.08.2009

Die Aufklärung des Repressionsmechanismus des Transkriptionsfaktors CcpN aus *B. subtilis* wird in dieser Publikation beschrieben. Mittels EMSA, „open complex formation“-Assays und *in vitro*-Transkription konnte gezeigt werden, dass CcpN am *gapB*-Promotor die die abortive Transkription inhibiert, während es am *pckA*- und *sr1*-Promotor die Bildung eines Elongationskomplexes verhindert. Weiterhin wurde eine spezifische Interaktion von CcpN und der α-Untereinheit der RNA-Polymerase detektiert, welche mit hoher Wahrscheinlichkeit für die Repression am *sr1*- und *pckA*-Promotor verantwortlich ist.


Manuskript IV
Rita A. Eckart, Sabine Brantl & Andreas Licht

Search for additional targets of the transcriptional regulator CcpN from *Bacillus subtilis*.

Akzeptiert bei *FEMS Microbiology Letters* am 31.07.2009

Bakterienspezies zeigte außerdem, dass auch in diesen eine CcpN-vermittelte Regulation von \( thyB \) wahrscheinlich ist.

3. Transcriptional repressor CcpN from *Bacillus subtilis* compensates asymmetric contact distribution by cooperative binding

(Manuskript I)

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Transcriptional Repressor CcpN from *Bacillus subtilis* Compensates Asymmetric Contact Distribution by Cooperative Binding

Andreas Licht* and Sabine Brantl

**Introduction**

Although many bacteria, including *Bacillus subtilis*, are able to utilise a vast number of other nutrients, glucose is their preferred carbon source. Therefore, cells need to shut-down other catabolic pathways in the presence of glucose to maximise the energy yield. This is accomplished by so-called catabolite repression. In *Escherichia coli*, catabolite repression is mediated by the central signalling molecule cAMP and its receptor protein CRP. By contrast, *B. subtilis* does not encode a CRP homologue nor does it produce detectable amounts of cAMP under aerobic conditions. Instead, catabolite repression in *B. subtilis* is carried out mainly by the major regulator CcpA. In contrast, sugar-dependent repression of three genes, *sr1* encoding a small untranslated RNA, and two genes, *gapB* and *pckA*, coding for gluconeogenic enzymes is mediated by the recently identified transcriptional repressor CcpN. Since previous DNase I footprinting yielded only basic information on the operator sequences of CcpN, chemical interference footprinting studies were performed for a precise contact mapping. Methyl interference, potassium permanganate and hydroxylamine footprinting were used to identify all contacted residues in both strands of the three operator sequences. Furthermore, ethylation interference experiments were performed to identify phosphate residues essential for CcpN binding. Here, we show that each operator has two binding sites for CcpN, one of which was always contacted more strongly than the other. The three sites that exhibited close contacts were very similar in sequence, with only a few slight variations, whereas the other three corresponding sites showed several deviations. Gel retardation assays with purified CcpN demonstrated that the differences in contact number and strength correlated well with significantly different $K_D$ values for the corresponding single binding sites. However, quantitative DNase I footprinting of whole operator sequences revealed cooperative binding of CcpN that, apparently, compensated the asymmetric contact distribution. Based on these data, possible consequences for the repression mechanism of CcpN are discussed.

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**Keywords:** CcpN; transcriptional repressor; chemical footprinting; carbon catabolite repression; DNA-protein-interaction
Krebs cycle intermediates, PEP carboxykinase, which catalyses the conversion of oxaloacetate to phosphoenolpyruvate. The ccpN gene is cotranscribed with the yqfL gene, resulting in a bicistronic mRNA. It was shown that this operon is not autoregulated, but constitutively expressed under both glycolytic and gluconeogenic conditions. Homologues of CcpN have been found in the genomes of other Bacilli, e.g. B. halodurans, B. cereus, B. anthracis and Geobacillus stearothermophilus, and in different Firmicutes. Recently, a third gene regulated by CcpN, sr1, has been discovered. This gene codes for a small untranslated RNA, SR1, which has been identified by a systematic search for small RNAs within intergenic regions of the B. subtilis genome. sr1 was expressed during gluconeogenesis, but repressed under glycolytic conditions. The trans-acting factor responsible for sugar-mediated repression was identified as CcpN. Previous DNase I footprinting experiments for all three known CcpN operators indicated different locations of the binding regions relative to the transcription start site.

The aim of the present work was to investigate the interaction between CcpN and its operator regions in more detail using chemical interference footprinting. These experiments showed that contact strength varied greatly, depending on the sequence of a given site. Gel retardation assays with single binding sites confirmed these observations. However, quantitative DNase I footprinting experiments with DNA fragments of all three genes spanning the operator showed a similarly asymmetric contact distribution, but here, contacts were concentrated upstream of the −35 region and the −10 regions of pckA and gapB operator, respectively. More-
Figure 1. Methylation interference of the sr1, pckA, and gapB operators. (a) C/T, Maxam-Gilbert C+T sequencing reaction; C, control (protein-free methylated DNA, this lane is equivalent to a Maxam-Gilbert G > A sequencing reaction); B and U, bound and unbound fraction of methylated DNA subjected to binding with CcpN-His. The numbers in the gels and column diagrams show the positions of the corresponding nucleotides relative to the transcription start site. Binding sites I and II for CcpN have been denoted according to interference footprinting experiments. Close contacts are indicated by black and grey triangles for G and A, respectively. (b) Column diagrams indicating the relative strength of interference signals for both strands of the three operators. Only positive signals, i.e., signals that indicate contacts, are shown. Measured values are averaged from four independent experiments.
residues within the three operators. KMnO₄, a strong oxidising agent, specifically oxidises thymine, thus impeding protein contacts. In addition to thymine, guanine is modified by KMnO₄, which results in bands for guanine residues in the gels. Figure 2 shows the positions of the modified thymine interfering with CcpN binding. In general, contact distribution correlated well with that found by methylation interference footprinting. The sr1 operator exhibited the following strong interference signals in binding site I: T_{−52} and T_{−50} at the top strand and T_{−48}, T_{−46}, T_{−44} at the bottom strand. However, in contrast to the contacts to guanine and adenine, contacts to thymine (T_{−22} and T_{−20} at the top strand) were slightly closer in binding site II. Significant contacts in binding site II have not been found on the bottom strand.

Both in the pckA and gapB operators, the positions of contacted thymine residues corresponded perfectly to those identified for guanine and adenine by methylation interference, too. The focus of contacts was in site II in the case of pckA (five close contacts, see Figure 2, at the top strand and T_{−12} and T_{−10} at the bottom strand), while only less close contacts were found in binding site I at the top strand and no significant contact at the bottom strand. The gapB operator exhibited strong interference signals only in site I (mainly T_{−16} and T_{−14} on the top strand and T_{−12} and T_{−10} on the bottom strand), whereas only less close contacts were found in binding site II at the top strand and no significant contact at the bottom strand. In all three operators, thymine bases that showed the strongest interference signals were located next to contacted guanine bases, together forming the contact center within each binding site.

**Hydroxylamine footprinting**

NH₂OH footprinting was used to analyse CcpN contacts to cytosine in the three operators. Hydroxylamine, a strong reductive agent, causes ring opening specifically at cytosine bases and, in this way, interferes with contact formation between protein and DNA. Figure 3 shows that contacts to cytosine bases were found in all three operators; however, the contacts were less close compared to the three other bases. Interference signals of almost equal intensity were found in the sr1 operator in site I (C_{−45} at the top strand and C_{−53} and C_{−51} on the bottom strand) and in site II (C_{−17} at the top strand and C_{−21} and C_{−19} on the bottom strand). The pckA operator showed only two contacted cytosine bases in binding site II and no contact in binding site I. Interestingly, in the gapB operator, three contacted

Figure 2. KMnO₄ interference of the sr1, pckA and gapB operators. (a) G/A, Maxam–Gilbert G>A sequencing reaction; C/T, Maxam–Gilbert C+T sequencing reaction; C, control (protein-free KMnO₄-treated DNA); B and U, bound and unbound fraction of methylated DNA subjected to binding with CcpN-His₅. The numbers in the gels and column diagrams show the positions of the corresponding nucleotides relative to the transcription start site. Binding sites I and II for CcpN are designated as in Figure 1. Close contacts are indicated by dark grey triangles. Only the gels for top and bottom strand of the sr1 operator are shown. (b) The column diagrams present the relative strength of interference signals for both strands of the three operators. Only positive signals, i.e. signals that indicate contacts, are shown. Measured values are averaged from four independent experiments.
cytosine bases were found in both site I (C\(_{-9}\) at the top strand and C\(_{-15}\) and C\(_{-17}\) at the bottom strand) and site II (C\(_{+22}\) at the top strand and C\(_{+17}\) and C\(_{+20}\) at the bottom strand). However, due to the weak nature of these interference signals, contacts to cytosine do not seem to play an important role in the CcpN–DNA interaction.

**Ethylation interference footprinting**

To determine phosphate groups of the DNA backbone contacted by CcpN, ethylation interference experiments were carried out. Figure 4 presents the positions at which ethylation interfered with CcpN binding. Both binding sites in the sr1 operator showed only two interference signals: In site I, T\(_{-50}\) at the top strand and A\(_{-47}\) at the bottom strand were contacted, and in site II, A\(_{-12}\) and A\(_{-15}\) at the bottom strand were contacted. In the pckA operator, contacts to the sugar-phosphate backbone were detected only in binding site II. Here, T\(_{-14}\) at the top strand and A\(_{-18}\) and T\(_{-10}\) at the bottom strand exhibited interference signals. The same was found for the gapB operator, where only binding site I showed two contacts, to T\(_{-14}\) at the top strand and T\(_{-10}\) at the bottom strand.

**Figure 3.** NH\(_2\)OH interference of the sr1, pckA and gapB operators (a) G/A, Maxam–Gilbert G>A sequencing reaction; C/T, Maxam–Gilbert C+T sequencing reaction; C, control (protein-free NH\(_2\)OH-treated DNA); B and U, bound and unbound fraction of methylated DNA subjected to binding with CcpN-His. The numbers in the gels and column diagrams show the positions of the corresponding nucleotides relative to the transcription start site. Binding sites for CcpN are designated as in Figure 1. Close contacts are indicated by white triangles. Only the gels for top and bottom strand of the sr1 operator are shown. (b) The column diagrams present the relative strength of interference signals for both strands of the three operators. As above, only positive signals are shown. Measured values are averaged from four independent experiments.

**Figure 4.** Ethylation interference of the sr1, pckA and gapB operators. The column diagrams present the relative strength of interference signals for both strands of the three operators. Only positive signals, i.e. signals that indicate contacts, are shown. Numbers in the column diagrams designate the positions of the corresponding nucleotides relative to the transcription start site. Measured values are averaged from three independent experiments.
Interestingly, the few DNA-backbone contacts were observed in most cases next to a contacted guanine residue. Obviously, these contacts play only a minor role in the binding of CcpN to its operators. Figure 5 summarises all probed contacts for the three operators.

**EMSA**

To determine the apparent equilibrium dissociation constants $K_D$ for the CcpN-DNA complex, 23 bp double-stranded oligonucleotides containing a single CcpN binding site were incubated with increasing concentrations of CcpN-His$_6$ (Figure 6(a)). $K_D$ values were estimated by non-linear regression using the average data from three independent experiments as described in Materials and Methods. The calculated $K_D$ values as well as the binding energy $\Delta G$ for the CcpN-DNA interaction for the single sites are summarised in Table 1. Binding energy was calculated with the help of Van’t Hoff’s reaction isobare $\Delta G = -RT \ln K$, where $R$ is the universal gas constant, $T$ is the absolute temperature (in Kelvin) and $K$ is the determined equilibrium association constant. The calculated $K_D$ values for the single binding sites corresponded very well to the contacts that were observed by interference footprinting: Binding site I of the gapB operator, the one with the most and closest contacts (see Figure 1), showed the lowest $K_D$ value, indicating a tight protein-DNA interaction, whereas binding site II of gapB or site I of pckA, both with less close contacts, exhibited high $K_D$ values. Determined dissociation constants ranged from as low as 98 nM (gapB, site I) till 4.4 $\mu$M (gapB, site II).

To test whether the equilibrium dissociation constants differ when using whole operators, double-stranded oligonucleotides containing both CcpN binding sites were incubated with increasing concentrations of CcpN-His$_6$ (Figure 6(b)). $K_D$ values were estimated by non-linear regression using the average data from three independent experiments as described in Materials and Methods. The apparent equilibrium dissociation constants were determined to be 19.3 nM, 15.5 nM and 12.8 nM for the sr1, pckA and gapB operators, respectively, and correspond well to the values determined by Servant et al. All operators showed significantly lower $K_D$ values than the single sites alone. At the sr1 operator, the average $K_D$ was decreased 30-fold, while the $K_D$ of site I of the pckA and site II of the gapB operators was decreased 160-fold and 340-fold, respectively.

**Figure 5.** Overview of the contacts in all three operators. (a) Overview of all contacts. Symbols used to indicate contacts to the bases are shown in the box below. Filled symbols denote close contacts (50%–100% compared to the strongest signal), open symbols represent medium or less close contacts (15%–50% compared to the strongest signal). For clarity, contacts with less than 15% relative strength are not shown. The $\sim$35 and $\sim$10 regions are boxed, and the transcription start site is indicated. Binding sites I and II are designated on the basis of all interference footprinting experiments. (b) Alignment of the core sequences of all binding sites. Positions that coincide with the consensus are shown in bold.
Quantitative DNase I footprinting

Since occupancy of single sites is not detectable in EMSA, the affinity of CcpN to the single sites within the complete operator was measured by quantitative DNase I footprinting. This technique allows us to determine $K_D$ values for site I and site II separately, even if they are located on one DNA.

Figure 6 (legend on opposite page)
The 400 bp oligonucleotides were incubated with increasing concentrations of purified CcpN-His in the same order as the binding sites appear in the DNase I footprinting gels. (b) EMSAs of whole CcpN operators. From left to right: 0; 5.2 nM; 7.8 nM; 11.7 nM; 17.6 nM; 26.3 nM; 39.5 nM; 59.3 nM; 88.9 nM; 133 nM; 200 nM. F, free DNA; operator, i.e. the occupation of both sites in the DNA complex. To allow for a direct comparison with (c), EMSAs for binding sites I and II are shown in Figure 6(c). In all experiments, the top strand was labelled, since the DNase I cleavage pattern of this strand was more homogeneous than that of the bottom strand. The degree of protection observed corresponded directly to the occupancy of the DNA by CcpN and allowed us to calculate the amount of complex formed. Apparent equilibrium dissociation constants were estimated by non-linear regression using the average data from three independent experiments. The calculated $K_D$ values, the Hill coefficients and the binding energy $\Delta G$ for the CcpN–DNA interaction for all single sites are summarised in Table 1. Interestingly, the apparent dissociation constants for the complete operator sequences differed from those found for the investigated single sites and from the results of the footprinting experiments.

### Cooperative Binding of CcpN

The $K_D$ values for each side in the context of the whole operator were in all cases lower than for the corresponding single sites alone (see Table 1). This was especially true for binding site I of pckA and binding site II of the gapB operator. The gain in free energy upon CcpN binding to two separated single sites was lower than to two sites in a complete operator, i.e. the occupation of both sites in the operator is cooperative. This was verified by the finding that the $K_D$ values obtained with DNA fragments spanning the whole operator are significantly lower than those obtained with single binding sites. Furthermore, when the values for the single site isotherms in the context of the whole operator were fit to the Hill equation (see Materials and Methods), the shape and the slope of the isotherms changed to a characteristic form for cooperative interactions. Moreover, the Hill coefficient $h$ is in each case >1 (see Table 1), which is a reliable sign for cooperativity. In the case of sr1, where the two binding sites have nearly identical $K_D$ values, the affinity of each site was increased by approximately equal amounts. By contrast, when one binding site was much stronger than the other, as in the case of pckA and gapB, the $K_D$ value for the weaker binding site was improved dramatically (from 4.4 μM to 114 nM for gapB site II), but the $K_D$ for the stronger binding site was mostly unaffected. A comparison of the binding isotherms for the single sites and the single-site isotherms for the complete operators that can be found in Figure 6(d) corroborates this conclusion.

#### Energetic calculations on CcpN-DNA interactions

Quantitative footprinting experiments like those described above were performed at 37°C and 52°C. The free energy $\Delta G$ was calculated on the basis of three independent experiments. Equation (1) describes the correlation between free energy ($G$), enthalpy ($H$) and entropy ($S$) and can be rearranged to yield equation (2), because $\Delta H$ and $\Delta S$ are independent of temperature. Thereby, $T_1$ and $T_2$ are

<table>
<thead>
<tr>
<th>Table 1. Apparent dissociation constants and free energies for all CcpN binding sites</th>
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<tbody>
<tr>
<td><strong>Single sites</strong></td>
</tr>
<tr>
<td><strong>Binding site</strong></td>
</tr>
<tr>
<td>sr1, site I</td>
</tr>
<tr>
<td>sr1, site II</td>
</tr>
<tr>
<td>pckA, site I</td>
</tr>
<tr>
<td>pckA, site II</td>
</tr>
<tr>
<td>gapB, site I</td>
</tr>
<tr>
<td>gapB, site II</td>
</tr>
</tbody>
</table>

Values were derived from three independent experiments. $\Delta \Delta G (\Delta G_{\text{complex}} - \Delta G_{\text{single}})$ is the extra free energy that is gained when the two occupied sites are together on one DNA molecule, and $h$ is the Hill coefficient.
310.15 K and 325.15 K, respectively, and $G_1$ and $G_2$ the free energies at the corresponding temperatures:

$$\Delta G = \Delta H - T \Delta S$$

$$\Delta S = \frac{\Delta G_1 - \Delta G_2}{T_2 - T_1}$$

Enthalpic and entropic contributions to CcpN-DNA binding were calculated using equation (2) and are summarised in Table 2. The CcpN-DNA interaction shows a small but unfavourable change in entropy that is overcome by a strong enthalpic contribution. This combination of enthalpy and entropy ensures that the CcpN-DNA interaction has nearly the same efficiency at all temperatures that are tolerated by B. subtilis.

**Discussion**

CcpN binds asymmetrically to its two consecutive binding sites in all three operators

Here, we report the high-resolution contact probing of the transcriptional repressor CcpN bound to its operator sites. CcpN, which has been identified recently as a repressor active under glycolytic conditions, is known to regulate three genes in B. subtilis: sr1, encoding a small untranslated RNA,13 and genes for two gluconeogenic enzymes, pckA and gapB.11 Using chemical interference footprinting with different chemical probes, we determined the bases contacted by CcpN in all three operators (summarised in Figure 5).

In all cases, two binding sites were identified, one of which was always contacted more strongly than the other. In the following, this site is referred to as the strong site, whereas the other is designated the weak site. Within all binding sites, core regions can be defined that resemble the consensus binding sequence TGTG(Y/A)YATAC that was previously determined for CcpN.13 A comparison of all core regions with this consensus is presented in Figure 5(b).

In the sr1 operator, the upstream binding site (site I, the strong site) was found to be contacted in a slightly stronger manner, but both binding sites showed extensive contacts especially to guanine and thymine residues (Figure 5) and less close contacts to adenine and cytosine residues, and to the sugar-phosphate backbone (Figures 1, 3 and 4). Moreover, both core regions conform well to the consensus. By contrast, contact distribution was found to be completely different in the other two operators. In the case of pckA, the majority of contacts were concentrated in the downstream binding site (site II, the strong site), where close contacts to all bases except cytosine were found (Figure 5). At site I, the weak site, only few and less close contacts were detected. Whereas the core region of the strong site again corresponded well to the consensus sequence, the core of the weak binding site deviated significantly from the consensus. Furthermore, although only one extended site appeared in the pckA DNase I footprint,11 chemical interference revealed that the pckA operator consists of two binding sites, too.

Similar results have been found for the gapB promoter, except that the upstream site (site I) proved to be the strong site. As in the case of pckA, the most and the closest contacts were found in the strong site in the consensus-like core region, whereas site II showed only a low level of similarity to the consensus sequence. A series of gapB operator mutants tested by Servant et al.13 can be evaluated in the light of the data published here: They found that a $T_{–14}$→A mutation, located in the strong site, severely inhibited CcpN binding, which can be explained by the close contact to the adenine residue on the complementary strand that we observed. Moreover, this position was shown to be invariant in the previously determined consensus sequence.13 This holds true for the $A_{230}$→G mutation too, which concerns an invariant base in the weak site. However, the observed effect was not that pronounced, since the contribution of this position is not that great in this case. By contrast, the $T_{–14}$→G mutation showed almost no effect on the CcpN-DNA interaction, despite the close contacts that we mapped for this position. However, this site has been shown to be more variable in the consensus sequence,13 and one could imagine that a mutation at this site is compensated by the surrounding sequence.

In all three operators, the major contacts determined with interference footprinting were contacts to guanine and thymine residues, and all focused within a core binding region. Since guanosine is methylated at N7 in the major groove, one can conclude that CcpN contacts its operator sequences primarily, but not exclusively, through contacts in the major groove, as found previously for many other proteins, e.g. RhaS from E. coli.14 Like transcription factor TyrR from E. coli,15 CcpN contacts its target through a large number of bases. Contacts to the sugar-phosphate backbone make only minor contributions to the CcpN-DNA interaction and, thus, do not seem to play an important role. Most probably, extended contacts to bases relieve the necessity to interact with the sugar-

**Table 2. Reaction enthalpy and entropy for the CcpN-DNA interaction**

<table>
<thead>
<tr>
<th>Binding site</th>
<th>$\Delta G$ 37°C (kJ/mol)</th>
<th>$\Delta G$ 52°C (kJ/mol)</th>
<th>$\Delta H$ (kJ/mol)</th>
<th>$\Delta S$ (kJ/(mol K))</th>
</tr>
</thead>
<tbody>
<tr>
<td>sr1, site I</td>
<td>–42.3</td>
<td>–41.8</td>
<td>–51.5</td>
<td>–0.03</td>
</tr>
<tr>
<td>sr1, site II</td>
<td>–42.7</td>
<td>–41.5</td>
<td>–68.4</td>
<td>–0.08</td>
</tr>
<tr>
<td>pckA, site I</td>
<td>–40.4</td>
<td>–39.5</td>
<td>–59.2</td>
<td>–0.06</td>
</tr>
<tr>
<td>pckA, site II</td>
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<td>–41.4</td>
<td>–48.6</td>
<td>–0.02</td>
</tr>
<tr>
<td>gapB, site I</td>
<td>–41.7</td>
<td>–41.3</td>
<td>–49.8</td>
<td>–0.03</td>
</tr>
<tr>
<td>gapB, site II</td>
<td>–41.2</td>
<td>–38.4</td>
<td>–100.1</td>
<td>–0.18</td>
</tr>
</tbody>
</table>

Quantitative footprinting was performed at 37°C and 52°C with DNA fragments carrying the whole operator sequence. The values were derived from three independent experiments.

"Manuskript I"
phosphate backbone. Interestingly, contacts to the sugar–phosphate backbone were found mostly downstream from one of the guanine bases that provided one of the main contacts.

The occurrence of two binding sites with different contact strengths within one operator is rather peculiar, as many proteins with two binding sites bind these sites with more-or-less equal affinity. In this regard, however, CcpN shows similarities with PurR, whose operators have one strong and one weak binding site, too, although the differences are not as pronounced as in the case of CcpN.

CcpN binding sites are located at different positions at each operator

Previous DNase I footprinting experiments indicated that the binding site distribution is different among the three CcpN-regulated promoters. Here, we substantiated these findings and determined the exact borders of the single binding sites using chemical interference footprinting experiments. Figure 5 shows that in all three operators, CcpN binding sites are located at different positions relative to the transcription start site.

At the sr1 operator, site I was found to be centred upstream of the −35 box, around −48, and site II centred around −19. Bases within the −35 box were not contacting by CcpN, and only one base of the −10 box exhibited one less close contact. In contrast, in the pckA operator, site I overlapped the −35 box partially and site II the −10 box completely. The gapB operator revealed another positioning of the binding sites. Here, binding site I covered the −10 box as does site II in the case of pckA, and site II was located downstream from the transcription start site with its centre at position +19.

Diverse distribution of operator sites is not an uncommon feature. Beside transcription factors that show conserved binding site positioning, like CytR from E. coli, numerous transcription factors bind to operators that are located at varying positions with regard to the promoter, as does CcpN. One example is CcpA, the major factor for carbon catabolite repression in B. subtilis, whose binding sites, termed cre elements, can be positioned differently relative to the transcription start site. Depending on their regulated gene, they are found at e.g., −33, −3 or +37. Interestingly, all these cre elements mediate transcriptional repression, although their respective repression mechanism has not been elucidated.

Based on the distribution of the CcpN binding sites at the three different promoters, it is tempting to speculate about different repression mechanisms. In the case of sr1, neither the −35 nor the −10 box are covered or contacted by CcpN. This might allow RNA polymerase to bind simultaneously with CcpN to the sr1 promoter, which would exclude repression by steric hindrance and could result in inhibition of open complex formation, e.g. as found for the MerR repressor of E. coli. Another conceivable mechanism is inhibition of promoter clearance, as shown for protein P4 of phage ϕ29 at the viral A2c promoter. In contrast, at the pckA and gapB promoters, inhibition of transcription might occur by steric hindrance of RNAP binding, since at least one binding site of these promoters completely covers the −10 box, as it is the case for the Fur protein from E. coli as well as many other transcriptional repressors. Future experiments will focus on the elucidation of the repression mechanism of CcpN at all three operators, for which the ligand that modulates CcpN activity still needs to be identified.

CbpN binds cooperatively to its two binding sites

Our interference footprinting experiments indicated that CcpN contacts its respective binding sites with different strengths, especially at the pckA and gapB promoters (Figure 6). These results were confirmed by EMSAs using oligonucleotides carrying the single binding sites. The $K_D$ values determined varied greatly from as low as 98 nM for the strong site of gapB to 4.4 μM for the associated weak site. The same was true for the strong and the weak site of the pckA operator, whereas the $K_D$ values for the binding sites of the sr1 operator did not differ much with 420 nM and 650 nM for site I and II, respectively, due to the only slight differences in contacts between these sites (Figure 6(a) and Table 1). Surprisingly, the $K_D$ values obtained in EMSAs with DNA fragments containing the whole operators were greatly reduced, up to 340-fold for the weak site of gapB, compared to those for the single binding sites and correlated well to what was found by Servant et al. Obviously, two binding sites on one DNA strand dramatically increase the binding efficiency of CcpN.

The determination of the $K_D$ values for the single site in the context of the complete operators confirmed these results. Here, in all three operators, both sites were occupied with almost the same efficiency and showed only slight variations in $K_D$ values between the strong and the weak sites. In addition, all $K_D$ values were, partly significantly, decreased. In the sr1 operator, both binding sites showed an almost equal increase in binding affinity, whereas at the pckA and gapB operators, only the weak binding sites exhibited a significantly lower $K_D$ in the complete operator. Thereby, the affinity of the strong binding sites was mainly unchanged or increased only slightly. This increase in binding affinity leads to an increase in energy gain upon CcpN binding: Binding to two sites that are in close vicinity on one DNA strand is energetically more favourable than binding to two separate strands. Furthermore, a change in the shape and slope of the binding curves, resulting from a Hill coefficient $>1$, which indicates cooperativity, was observed. All this leads to the conclusion that CcpN apparently binds to its operators in a cooperative way, but this cooperativity is different for the three promoters. While the sr1 operator shows two more-or-less equal
binding sites, the \textit{pckA} and \textit{gapB} operators are composed of one main and one auxiliary site, and binding to the auxiliary site was found to be greatly improved in the presence of the main site. Strong and weak binding sites were observed also for the \textit{DeoR} repressor–operator system in \textit{B. subtilis}.\textsuperscript{28} The \textit{DeoR} operator consists of one full and one half binding site but, unlike \textit{CcpN}, \textit{DeoR} does not bind single sites.

Cooperative binding suggests an interaction between the \textit{CcpN} molecules bound to the stronger and weaker sites. Conspicuously, the spacer region between the two binding sites differs between the three operators (see \textbf{Figures 5 and 7}). Whereas in the \textit{sr1} and \textit{gapB} operator it comprises three helical turns, in the \textit{pckA} operator, only two helical turns separate the two binding sites. This indicates that, at least in the case of \textit{sr1} and \textit{gapB}, \textit{CcpN} most likely bends its operator DNA to enable a contact between the two binding entities.

\textbf{CcpN binding is driven exclusively by a strong binding enthalpy}

The determination of binding constants and free binding energy showed that \textit{CcpN} binding to its operator is unfavourable in terms of entropy change, i.e. entropy decreases upon \textit{CcpN}–DNA interaction. This effect is overcome by a strong favourable enthalpic contribution, likely due to the numerous and close contacts made to the bases in the operator sequence. This has a clear practical consequence for \textit{B. subtilis}: Since this species tolerates temperatures from as low as 12 °C to as high as 52 °C, a strong binding enthalpy, which is temperature-independent, and a low binding entropy change, whose contribution to total binding energy depends on the temperature, ensures that \textit{CcpN} retains its binding affinity and \textit{K_D} value for its operators over a large temperature scale.

\textbf{Figure 7.} Ribbon model of all \textit{CcpN} operators. Symbols used to indicate contacts between \textit{CcpN} and the operator are the same as in \textbf{Figure 5}. The binding sites and the core binding regions are indicated by brackets.
Materials and Methods

Preparation of labelled CcpN targets

Oligonucleotides were purified by treatment with piperidine for 30 min at 90 °C to avoid contamination with depurinated DNA resulting from the removal of the protective groups during synthesis. Subsequently, purified oligonucleotides were 5′ end-labelled with [γ-32P]ATP using bacteriophage T4 polynucleotide kinase (NEB) and purified from denaturing 15% (w/v) polyacrylamide gels. Pairwise combinations of labelled and unlabelled oligonucleotides were annealed by incubation at 65 °C for 5 min and subsequent slow cooling to 37 °C. The top and bottom strand of all oligonucleotides carry two G or C residues, respectively, at each end to facilitate correct annealing and to promote additional stability. Labelled double-stranded DNA fragments for the EMSAs with the whole operator sequences were obtained by PCR using the appropriate primer pairs (all oligonucleotides used in this study are summarised in Table 3). The PCR products were purified from an ethidium bromide-stained native 6% polyacrylamide gel and 5′ end-labelled as described above. The DNA was then separated from unincorporated [γ-32P]ATP by passage through a Sephadex column.

Over-expression and purification of CcpN

A ccpN over-expression strain was constructed by cloning a NcoI/BglII digested PCR fragment obtained with primers SB673 and SB674 on chromosomal DNA of B. subtilis DB104 into the pQE60 NcoI/BglII vector (Qiagen). The resulting vector was designated pQGDR. For cloning a NcoI/BglII digested PCR fragment obtained with primers SB673 and SB674 on chromosomal DNA of B. subtilis with primers SB673 and SB674 on chromosomal DNA of B. subtilis

The resulting vector was designated pQGDR. For cloning and subsequent purification of the C-terminally His-tagged protein, E. coli strain TG1(REP4) was used. The sequence was confirmed using a Sequenase kit (Amersham Bioscience).

A TG1(REP4, pQGDR) overnight culture grown in TY medium with 1 mM IPTG was used. The 5′ end-labelled DNA fragments were modified by dimethyl sulphate as described for the G→A reaction using the Merck oligonucleotide sequencing kit. Modified DNA was subjected to CcpN binding and EMSA as described above. Bound and unbound fractions were separated on a native 6% polyacrylamide gel and visualised by wet autoradiographic exposure. Bound and unbound DNA was cut out and eluted from the gel by diffusion (elution buffer: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.15 M NaCl), treated with phenol/chloroform and precipitated in ethanol. DNA samples and protein-free DNA as control were depurinated for 15 min at 90 °C, cleaved by piperidine for 30 min at 90 °C, precipitated in ethanol twice, resuspended in formamide loading dye and separated on a 15% polyacrylamide sequencing gel.

Potassium permanganate interference footprinting

The 5′ end-labelled DNA fragments were modified by dimethyl sulphate as described for the G→A reaction using the Merck oligonucleotide sequencing kit. Modified DNA was subjected to CcpN binding and EMSA as described above. Bound and unbound fractions were separated on a native 6% polyacrylamide gel and visualised by wet autoradiographic exposure. Bound and unbound DNA was cut out and eluted from the gel by diffusion (elution buffer: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.15 M NaCl), treated with phenol/chloroform and precipitated in ethanol. DNA samples and protein-free DNA as control were depurinated for 15 min at 90 °C, cleaved by piperidine for 30 min at 90 °C, precipitated in ethanol twice, resuspended in formamide loading dye and separated on a 15% polyacrylamide sequencing gel.

Hydroxylamine interference footprinting

The 5′ end-labelled DNA fragments were modified by dimethyl sulphate as described for the G→A reaction using the Merck oligonucleotide sequencing kit. Modified DNA was subjected to CcpN binding and EMSA as described above. Bound and unbound fractions were separated on a native 6% polyacrylamide gel and visualised by wet autoradiographic exposure. Bound and unbound DNA was cut out and eluted from the gel by diffusion (elution buffer: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.15 M NaCl), treated with phenol/chloroform and precipitated in ethanol. DNA samples and protein-free DNA as control were depurinated for 15 min at 90 °C, cleaved by piperidine for 30 min at 90 °C, precipitated in ethanol twice, resuspended in formamide loading dye and separated on a 15% polyacrylamide sequencing gel.

Ethylation interference footprinting

The 5′ end-labelled DNA fragments were modified by dimethyl sulphate as described for the G→A reaction using the Merck oligonucleotide sequencing kit. Modified DNA was subjected to CcpN binding and EMSA as described above. Bound and unbound fractions were separated on a native 6% polyacrylamide gel and visualised by wet autoradiographic exposure. Bound and unbound DNA was cut out and eluted from the gel by diffusion (elution buffer: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.15 M NaCl), treated with phenol/chloroform and precipitated in ethanol. DNA samples and protein-free DNA as control were depurinated for 15 min at 90 °C, cleaved by piperidine for 30 min at 90 °C, precipitated in ethanol twice, resuspended in formamide loading dye and separated on a 15% polyacrylamide sequencing gel.

EMSA and determination of apparent equilibrium dissociation constant KD

Binding reactions were performed in a final volume of 10 μl containing 0.5× TBE, 0.05 g/l of herring sperm DNA as non-specific competitor, 1 nM end-labelled DNA fragment and 5.2 nM to 17.7 μM CcpN-His6. All CcpN-His6 dilutions were made in storage buffer and the same volume of diluted protein was used in each sample to ensure an equal concentration of salt. After incubation at 37 °C for 15 min, the reaction mixtures were separated on native 6% (for whole operator DNA fragments) or 8% (for 23 bp DNA fragments) polyacrylamide gels run at room temperature for 1 h at 200 V. Visualisation and quantification of the bands were performed using a Fuji-PhosphorImager and the PCUBAS 2.09 quantification software (Raytest). All autoradiograms were made from dried gels. The image data generated by scanning the gel are linear proportionally to the radiation intensity of the sample. The amount of CcpN–DNA complex relative to the concentration of CcpN was fit with the non-linear regression programme Solver (included in Microsoft® Excel) to the following equation:

\[
[C] = \frac{[D][P]}{K_D + [P]}
\]

where [C], [D] and [P] represent total concentrations of formed complex, DNA and protein, respectively, and KD is the apparent equilibrium dissociation constant.
Table 3. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB499</td>
<td>5′ GGAAAATGTGTTATACAGTTTGG</td>
<td>sr1, site I, upper strand</td>
</tr>
<tr>
<td>SB500</td>
<td>5′ CCAACCTGTATACACATTTTCC</td>
<td>sr1, site I, lower strand</td>
</tr>
<tr>
<td>SB964</td>
<td>5′ GTAAATGTCATATTATACAGTTTGG</td>
<td>sr1, site II, upper strand</td>
</tr>
<tr>
<td>SB965</td>
<td>5′ CCATTAATATGTCACATTTTCC</td>
<td>sr1, site II, lower strand</td>
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<tr>
<td>SB962</td>
<td>5′ GGAATATATGTTATACTAATTGG</td>
<td>pckA, site I, upper strand</td>
</tr>
<tr>
<td>SB963</td>
<td>5′ CCATTAATATGTCACATTTTCC</td>
<td>pckA, site I, lower strand</td>
</tr>
<tr>
<td>SB960</td>
<td>5′ GGAATATATGTTATACTAATTGG</td>
<td>pckA, site II, upper strand</td>
</tr>
<tr>
<td>SB961</td>
<td>5′ CCATTAATATGTCACATTTTCC</td>
<td>pckA, site II, lower strand</td>
</tr>
<tr>
<td>SB969</td>
<td>5′ GGAATATATGTTATACTAATTGG</td>
<td>gapB, site I, upper strand</td>
</tr>
<tr>
<td>SB970</td>
<td>5′ CCATTAATATGTCACATTTTCC</td>
<td>gapB, site I, lower strand</td>
</tr>
<tr>
<td>SB988</td>
<td>5′ GGAATATATGTTATACTAATTGG</td>
<td>gapB, site II, upper strand</td>
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<tr>
<td>SB887</td>
<td>5′ CCATTAATATGTCACATTTTCC</td>
<td>gapB, site II, lower strand</td>
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<td>SB886</td>
<td>5′ GGAATATATGTTATACTAATTGG</td>
<td>gapB, site II, lower strand</td>
</tr>
<tr>
<td>SB985</td>
<td>5′ CCAATTATAGGATCGATTTTCC</td>
<td>sr1, complete operator, upper strand</td>
</tr>
<tr>
<td>SB867</td>
<td>5′ CTGCAGAGATCTTTATTAGTGATGGTGATGGTGTAGGATTTCATTTTCAGA</td>
<td>Plasmid pQGDR</td>
</tr>
<tr>
<td>SB870</td>
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<td>Plasmid pQGDR</td>
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<td>sr1 downstream primer</td>
</tr>
<tr>
<td>SB423</td>
<td>5′ TCGAGGATCAGATCGATCGTTTACAGTTAATGATGAT</td>
<td>sr1 upstream primer</td>
</tr>
<tr>
<td>SB1027</td>
<td>5′ CCAATTATAGGATCGATTTTCC</td>
<td>gapB downstream primer</td>
</tr>
<tr>
<td>SB1028</td>
<td>5′ CCAATTATAGGATCGATTTTCC</td>
<td>gapB downstream primer</td>
</tr>
<tr>
<td>SB1029</td>
<td>5′ CCAATTATAGGATCGATTTTCC</td>
<td>pckA upstream primer</td>
</tr>
<tr>
<td>SB1030</td>
<td>5′ CCAATTATAGGATCGATTTTCC</td>
<td>pckA downstream primer</td>
</tr>
</tbody>
</table>

Oligonucleotides SB673 and SB674 were used to construct plasmid pQGDR. SB342, SB423 and SB1027-SB1030 were used as primers for the amplification of whole operator fragments. All other oligonucleotides were annealed pairwise to create double-stranded targets for footprinting experiments and EMSAs.
described above. Bound and unbound fractions were separated on a native 6% polyacrylamide gel and isolated as described above. The DNA was cleaved with 143 mM NaOH at 90 °C for 30 min as described.\textsuperscript{32} Protein-free DNA as a control was prepared by NaOH cleavage of an aliquot of the ethylated DNA. After precipitation in ethanol twice and resuspension in formamide loading dye, the samples were separated on a 15% polyacrylamide sequencing gel.

**Densitometric quantification of the footprinting experiments**

Band intensities were determined with quantification software (PCBAS 2.09, Raytest) and, afterwards, normalised by dividing them by the total band intensity of the unbound DNA for each base position. Negative values were interpreted as interference signals.

**Quantitative DNase I footprinting**

DNase I footprinting was performed in a final volume of 10 µl containing 0.5× TBE, 6.25 mM MgCl₂, 0.05 g/l of herring sperm DNA, 1 nM end-labelled DNA fragment and 8.1 nM to 5.9 nM CcpN-Hiss. After incubation at 37 °C for 30 min, the samples were treated with 1 µl of DNase I (Roche, 0.05 U/µl) for 2 min at 37 °C. Two control samples, one without protein, one without DNase I, were treated in parallel. The reaction was stopped by extraction with phenol and subsequent precipitation in ethanol. The pellets were dissolved in 3 µl of formamide loading dye, denatured for 5 min at 90 °C and separated on a denaturing 15% polyacrylamide gel along with a Maxam-Gilbert sequencing reaction obtained from the same DNA fragment. The dried gel was analysed by PhosphorImaging. DNA occupancy by CcpN was determined by measuring the band intensity at the binding sites divided by the band intensity of the unbound DNA versus band intensity of the unbound DNA for each base position. The dried gel was analysed by PhosphorImaging. The dried gel was analysed by PhosphorImaging. The dried gel was analysed by PhosphorImaging.

To ensure that the CcpN–DNA complex is at equilibrium, footprinting experiments with different incubation times before DNase I cleavage were carried out. Steady state was reached no later than after 5 min of incubation. To show that DNase I is not able to displace CcpN from its operator, footprinting experiments with different concentrations of DNase I were performed. The amount of CcpN–DNA complex relative to the CcpN concentration was fitted with the non-linear regression programme Solver (included in Microsoft® Excel) to the Hill equation:

\[
[C] = \frac{[D]h}{K_h + [P]^h}
\]

where [C], [D] [P] and h represent total concentrations of formed complex, DNA, protein and the Hill coefficient, respectively, and \(K_h\) is the apparent equilibrium dissociation constant.

**Acknowledgements**

We thank E. Birch-Hirschfeld (Institut für Virologie, Jena) for synthesising the oligodeoxyribonucleotides. This work was supported by grant BR1552/6-1 from Deutsche Forschungsgemeinschaft (to S. B). A. L. is financed by a scholarship from the “Fonds der chemischen Industrie”.

**References**


Edited by M. Gottesman

(Received 21 July 2006; received in revised form 4 September 2006; accepted 6 September 2006)
Available online 12 September 2006
4. Identification of ligands affecting the activity of the transcriptional repressor CcpN from *Bacillus subtilis*.

(Manuskript II)

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Identification of Ligands Affecting the Activity of the Transcriptional Repressor CcpN from Bacillus subtilis

Andreas Licht¹*, Ralph Golbik² and Sabine Brantl¹

Carbon catabolite repression in Bacillus subtilis is mediated primarily by the major regulator CcpA. However, sugar-dependent repression of three genes, sr1 encoding a small nontranslated RNA and two genes coding for gluconeogenic enzymes, gapB and pckA, is carried out by the transcriptional repressor CcpN (control catabolite protein of gluconeogenic genes). It has previously been shown that ccpN is constitutively expressed, which leads to a constant occupation of all operators with CcpN. Since this would not allow for specific regulation, a ligand that modulates CcpN activity is required. In vitro transcription assays demonstrated that CcpN is able to specifically repress transcription to a small extent at the three mentioned promoters in the absence of an activating ligand. Upon testing of several ligands, including nucleotides and glycolysis intermediates, it could be shown that ATP is able to specifically enhance the repressing activity of CcpN, and this effect was more pronounced at a slightly acidic pH. Furthermore, ADP was found to specifically counteract the repressive effect of ATP. Circular dichroism measurements demonstrated a significant alteration of CcpN structure in the presence of ATP at acidic pH and in the presence of ADP. Electrophoretic mobility shift assays revealed that neither ATP nor ADP altered the affinity of CcpN for its operators. Therefore, we hypothesise that the effect of ligand-bound CcpN on the RNA polymerase might be due to a conformational switch that alters the interaction between the two proteins. Based on these results, a working model for CcpN action is discussed.

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Keywords: CcpN; transcriptional repressor; in vitro transcription; carbon catabolite repression; CD spectroscopy

Abbreviations used: CcpN, control catabolite protein of gluconeogenic genes; EMSA, electrophoretic mobility shift assay; RNAP, RNA polymerase; CTP, cytidine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.

Introduction

Most bacteria, among them Bacillus subtilis, are able to use a huge variety of nutrients.¹,² Nonetheless, glucose is the preferred carbon source for most of them.³ This requires other catabolic pathways to be shut down in the presence of glucose to maximise their energy yield. This process of catabolite repression in B. subtilis is mediated mainly by the concerted action of CcpA and HPr-Ser46-P, which can interact to form a transcriptional regulator.⁴ Though the majority of genes involved in carbon metabolism are regulated by the CcpA/HPr system, at least three genes, gapB, pckA and sr1, are downregulated in the presence of glucose by an alternative transcriptional repressor named CcpN (control catabolite protein of gluconeogenic genes), which exerts its function under glycolytic conditions.⁵-⁷ gapB and pckA encode enzymes that are exclusively active during gluconeogenesis,⁸ while sr1 codes for a small untranslated RNA, which has been identified by a systematic search for small RNAs within intergenic regions of the B. subtilis genome.⁹ The sr1 gene was also found to be expressed during gluconeogenesis but repressed under glycolytic conditions. Its gene product, SR1, inhibits translation initiation of ahrC mRNA, encoding a transcriptional activator of the arginine catabolic operons, by a novel mechanism. Seven regions of complementarity between SR1 and ahrC mRNA have been found, designated A to G. Upon SR1/ahrC mRNA interaction, structural alterations

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Received 7 February 2008; received in revised form 29 April 2008; accepted 2 May 2008
Available online 8 May 2008

Edited by J. Karn

doi:10.1016/j.jmb.2008.05.002
are induced between the ahrC ribosome binding site and region G located 90 nt downstream from it. These structural alterations prevent the binding of the 3OS ribosomal subunit.10,11

The ccpN gene forms a bicistronic operon with the yqfL gene, whose function is not yet fully clear. This operon is not autoregulated but constitutively expressed under both glycolytic and gluconeogenic conditions.7 Homologues of CcpN have been found in the genomes of other bacilli, for example, Bacillus halodurans, Bacillus cereus, Bacillus anthracis and Geobacillus stearothermophilus, and in different firmicutes.7

Recent investigations have demonstrated that CcpN occupies two distinct binding sites at each of the three regulated promoters. The position of the operator sites with respect to the transcriptional start site varies depending on the promoter, but in each case, one of these sites is contacted more efficiently than the other one. However, it has been shown that both binding sites are bound with equal affinity when located in close vicinity, since CcpN binds its half-sites in a cooperative manner.12

The aim of the present work was to identify the ligands that modulate the activity of CcpN. Electrophoretic mobility shift assays (EMSAs) demonstrated that none of the investigated potential ligands altered the affinity of CcpN to its operator. Therefore, in vitro transcription assays with native B. subtilis RNA polymerase (RNAP) were used as an alternative method to investigate the influence of various substances on the repression activity of CcpN. These assays showed a specific increase in repression activity in the presence of high concentrations of ATP and at low pH, whereas high concentrations of ADP were able to counteract the effect of ATP. Furthermore, circular dichroism (CD) measurements that revealed a substantial ATP-induced alteration of CcpN secondary structure have been performed. The combination of these data sets allowed to develop a new working model on the mechanism of action of CcpN.

Results

In vitro transcription experiments were performed with B. subtilis crude extracts from a CcpN knockout strain (DB104 ccpN::cat)7 that were filtrated through a Millipore column (molecular mass cutoff: 100,000 Da). This allows the separation of the RNAP holoenzyme from smaller proteins but retains any RNAP-associated factors. It has been confirmed previously that RNAP purified this way yields the same results as His-tagged B. subtilis RNAP purified according to the protocol of Fujita and Sadaie13 and as native B. subtilis RNAP prepared according to the protocol of Sogo et al. (data not shown).14

A ccpN/yqfL double knockout strain was complemented with a plasmid carrying the ccpN gene under control of pDEG to ensure that yqfL has no effect on the metabolic regulation of the sr1 gene. Since the ccpN gene itself is not regulated,7 this strain—after proper induction—behaves as a yqfL single knockout strain. Northern blot analyses revealed that this strain shows a response to glucose like the wild-type strain, although with a slightly reduced general sr1 transcription level (Fig. S1). This corresponds perfectly to the findings of Servant et al., who observed the same effects when investigating the influence of YqfL on gapB and pckA regulation.7 Therefore, all effects observed below can be attributed to the action of CcpN alone.

CcpN is able to specifically repress transcription at the sr1, gapB and pckA promoters

Linear DNA molecules carrying the sr1, gapB, pckA or RNAII and RNAIII promoter, respectively, were incubated with increasing concentrations of CcpN and used as a template for an in vitro transcription reaction to determine whether CcpN per se is able to repress transcription without the addition of a ligand. In vitro transcriptions were performed with B. subtilis RNAP for the sr1 and gapB promoters and—as a negative control—for promoters pII and pIII of streptococcal plasmid pPS10, controlling transcription of RNAII and RNAIII,15 respectively. Since B. subtilis RNAP yielded only very faint bands in the case of pckA, Escherichia coli RNAP was used instead. All key experiments with the sr1 promoter were performed with both polymerases to ensure that the results obtained with E. coli RNAP were comparable to those obtained with B. subtilis RNAP. Figure 6a shows the results, and Fig. 6 summarises all in vitro transcription experiments for better clarity. Once the CcpN concentration exceeded a certain threshold, all the promoters that are subject to regulation by CcpN in vitro revealed reduced transcription. By contrast, promoters pII and pIII, which are not subject to regulation by any B. subtilis protein, were not affected by CcpN even at very high concentrations. The observation that the sr1, gapB and pckA promoters are repressed by CcpN even in the absence of an added ligand corresponds very well to the observations made by Servant et al., who reported a significant derepression of the gapB and the pckA gene in a ccpN knockout strain.7

LacZ fusions show that different glycolysis mutants influence repression by CcpN

Since the presence of glucose in the medium influences CcpN activity, we constructed transcriptional sr1–lacZ fusions to investigate whether intermediates of the glycolytic pathway affect CcpN. These constructs were integrated into the chromosomes of B. subtilis strains that bear mutations in different glycolytic genes, thus interrupting glycolysis at certain steps. Strain QBS531 harbours a knockout of glucose-6-phosphate isomerase, and strain SU22 harbours a mutation in the glyceraldehyde-3-phosphate-dehydrogenase gene.15 Both strains showed growth curves similar to the wild type (data not shown). β-galactosidase measurements, summarised in Table 1, showed that in the wild-type strain, sr1 expression was repressed ≈ 37-
fold. Strain SU22 exerted a CcpN-mediated repression of factor 33, which is still significant but not as strong as the wild type. By contrast, strain QB5331 suffered from a severe lack in the ability to respond to CcpN in the presence of glucose in the medium, as it only showed a repression factor of 3.5. These results might imply that one of the glycolysis intermediates between glucose-6-phosphate and 1,3-bisphosphoglycerate is the ligand of CcpN. This hypothesis was surveyed in in vitro transcription assays.

**Carbon catabolism intermediates do not affect CcpN-mediated repression**

*In vitro* transcription assays were performed in the presence of a variety of substances, including nucleotides and carbon catabolism intermediates, to test whether certain molecules, especially glycolysis intermediates, affect the repression effect of CcpN. Since some intermediates are not commercially available and others are present at very low concentrations *in vivo*, only certain compounds were tested. A complete list of all tested substances can be found in Table 2. Since the experiments presented in Fig. 1 demonstrated that the three promoters respond to CcpN in the same manner, only the sr1 promoter was used as a model promoter for this screening. Figure 2 shows the results of these experiments. Of all tested nucleotides, only ATP had an effect on the transcription level. It increased transcription efficiency by a factor of 5, but this effect was not related to the presence of CcpN. This increase in the presence of 3 mM ATP can be explained by the increase in stability of the open complex, since an A is the first nucleotide of all three newly synthesised transcripts. Based on these results, all other substances were tested in the

![Fig. 1](image_url). Effect of CcpN and ATP on *in vitro* transcription. (a) *In vitro* transcription assay at pH 7.0 using 100 nM DNA fragment containing the promoter of sr1, gapB, pckA or RNAII/III at (a) 0.1 mM ATP or (b) 3 mM ATP. *B. subtilis* RNAP (100 nM) was used in each reaction. CcpN concentration from left to right was 0 nM, 78 nM, 156 nM, 313 nM, 625 nM and 1.25 μM, respectively. Where indicated, 50 nM *E. coli* RNAP was used. A radioactively labelled 89-nt DNA fragment served as a loading control to ensure equal amounts of the reaction being loaded onto each lane. The bands corresponding to the transcript (T) and to the loading control (L) are indicated. The autoradiograms of the gels are shown.

### Table 1. Results of β-galactosidase measurements

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>−Glucose (Miller units)</th>
<th>+Glucose (Miller units)</th>
<th>Repression factor</th>
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<tr>
<td>DB104</td>
<td>None</td>
<td>890 (±63)</td>
<td>24 (±4)</td>
<td>37</td>
</tr>
<tr>
<td>SU22</td>
<td>gapA</td>
<td>523 (±5)</td>
<td>16 (±5)</td>
<td>33</td>
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<tr>
<td>QB5331</td>
<td>pgi</td>
<td>359 (±89)</td>
<td>104 (±14)</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Summary of β-galactosidase measurements with wild-type *B. subtilis* and different strains with mutated glycolysis genes. Denoted mutations refer only to mutations in genes of the glycolytic pathway. Cultures were grown in SP medium to an OD600 of 2.0 (early stationary phase). Data are averaged from three independent experiments.
Table 2. List of all tested putative ligands in *in vitro* transcription

<table>
<thead>
<tr>
<th>Substance</th>
<th>Relative transcription</th>
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<tr>
<td>Control</td>
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<tr>
<td>CTP</td>
<td>0.8×</td>
</tr>
<tr>
<td>AMP</td>
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</tr>
<tr>
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</tr>
<tr>
<td>dAMP</td>
<td>1.5×</td>
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<td>Adenosine</td>
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</tr>
<tr>
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<td>GDP</td>
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<tr>
<td>GTP</td>
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<td>2-Phosphoglycerate</td>
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Summary of all substances investigated in *in vitro* transcription. Relative transcription shows the amount of transcript at 625 mM CcpN divided by the amount of transcript in the absence of CcpN. All substances were applied at 1 mM final concentration. Data are averaged from three independent experiments.

- AMP, adenosine monophosphate; ATP, adenosine triphosphate; GMP, guanosine monophosphate; GDP, guanosine diphosphate; dGTP, deoxyguanosine triphosphate; dATP, deoxyadenosine triphosphate; GTP, guanosine triphosphate; dTTP, deoxythymidine triphosphate; SAM, S-adenosyl-l-methionine.

Figure 2. Investigation of putative effectors. *In vitro* transcription assay at pH 7.0 using 100 nM DNA fragment containing the sr1 promoter. *B. subtilis* RNAP (100 nM) was used in each reaction. All investigated substances were applied at 1 mM final concentration. C, control; 1, glyceraldehyde-3-phosphate (free acid); 2, phosphoenolpyruvate; 3, dATP; 4, pyruvate; 5, citrate; 6, fructose-1,6-bisphosphate; 7, GDP. Table 2 shows a summary of all tested substances. The autoradiogram of the gel is shown.

The search for conserved domains in the CcpN sequence revealed, beside the DNA binding domain, a pair of CBS domains. These domains can be found in a variety of proteins in all three kingdoms of life and have been shown to exert different functions, such as binding of adenine nucleotides and formation of an oligomerisation interface or parts of an ion transport channel. Since binding of ATP or other adenine nucleotides would be very feasible in the case of CcpN, as it reflects the metabolic state of the cell, a series of experiments in the presence of ATP were performed. Since the results obtained at constant CcpN concentrations did not show a specific effect of ATP (Fig. 2), the CcpN concentration was varied. As can be seen in Fig. 1b, the presence of 3 mM ATP decreased the minimal inhibitory concentration of CcpN by approximately a factor of 2 at all three promoters. Regarding the efficient expression of these three genes *in vivo*, the effect was considerably smaller than expected. Therefore, it seemed that another ligand is required for efficient repression.

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**Acidic pH value is the second requirement for CcpN-dependent repression**

As shown in Fig. 2, low pH value in the presence of high ATP concentration led to a strong and specific repression of transcription by CcpN. To examine whether low pH value alone would be sufficient to induce CcpN-dependent repression, we performed *in vitro* transcription experiments at constant CcpN concentration in the presence of 0.1 mM ATP while pH was decreased from pH 7.2 to 6.5 (Fig. 3a). Alternatively, the effect of increasing CcpN concentration at pH 6.5 and 0.1 mM ATP was investigated (data not shown). Neither of these combinations showed any specific repression at all, implying that low pH is necessary but alone not sufficient for CcpN activity. A second set of experiments, using the same combinations of pH and CcpN concentration, but performed at 3 mM ATP, showed a strong specific repression effect (Figs. 3b and 4a–c). This indicates that a
combination of ATP and low pH is required to unfold the full repression capability of CppN.

ADP can specifically counteract the effect of ATP to relieve repression by CppN

Recently, the crystal structure of the regulatory domain of CppN was solved in the group of N. Declerck who showed that CppN is, besides binding ATP, also able to bind ADP (D. Chaix et al., manuscript in preparation). Inspired by this finding, we tested whether ADP had any influence on the repression activity of CppN in in vitro transcription assays. Figure 3c shows that ADP alone in addition to 0.1 mM ATP neither increased nor decreased CppN-mediated repression. However, when equimolar concentrations of ADP were added to an in vitro transcription reaction performed with 3 mM ATP at pH 6.5, ADP was capable to completely counteract the repression-enhancing effect of ATP (Fig. 4d–f).

CcppN mutated in a CBS domain loses its ability to respond to ATP or ADP

To examine whether CcppN with a mutation in its nucleotide binding domain retains its ability to respond to the two nucleotides, we investigated a mutant version of CcppN in in vitro transcription: K127A (Fig. 5). In this mutant, a conserved amino acid within one of the two CBS domains was replaced by an alanine. The mutant was a kind gift by Stéphane Aymerich and will be published elsewhere (D. Chaix et al., manuscript in preparation). Figure 6 shows that this mutant exerted the same kind of basic repression that could be observed with the wild type (Fig. 1) but lacked the ability to respond to ATP or ADP. According to Stéphane Aymerich (D. Chaix et al., manuscript in preparation), this mutant is not able to repress gapB or pckA transcription in vivo.

Repression conditions do not change the affinity of CcppN for DNA

Since ATP and low pH value have a strong effect on CcppN activity, we wanted to analyse whether they affect the affinity of CcppN for its operator sequence. To this end, a double-stranded DNA fragment harbouring the sr1 operator region was incubated with increasing concentrations of CcppN and subjected to an EMSA. This reaction was performed under nonrepressive conditions, in the presence of 3 mM ATP or in the presence of 3 mM ATP and a pH...
of 6.5 (Fig. 7). Both ATP and low pH, where applicable, were also present in the gel and in the running buffer to ensure that the conditions did not change during electrophoresis. However, none of the repression conditions affected the affinity of CcpN to its operators. This suggests that the specific repression of CcpN induced by ATP and an acidic pH shift is not based on an increased affinity to the promoter.

**CD measurements reveal an influence of ATP and ADP on the protein structure**

CD experiments were used to detect an influence of ATP or ADP on the secondary structure of CcpN. The far-UV CD spectra of the protein in the presence and absence of ATP or ADP at neutral or acidic pH are presented in Fig. 8. Without addition of a ligand, the protein displayed two negative extrema near 208 and 222 nm that indicate the presence of α-helical structures. At neutral pH, the spectrum did not alter significantly after addition of ATP. However, when ATP was added at pH 6.5, a substantial decrease in the α-helical content could be observed. This corresponds to the finding that only at acidic pH ATP was able to increase the repression efficiency of CcpN. On addition of increasing concentrations of ADP, a change in the CD spectrum of CcpN could also be observed, though not as pronounced as in
Interestingly, the ADP effect did not seem to depend on pH, as it was almost the same at neutral and acidic conditions.

Discussion

ATP and acidic pH were identified as the two factors required for the full repression capability of CcpN

In this study, we present the identification of ligands that are necessary for CcpN to work as an efficient repressor, as well as an investigation of ligand–protein interaction. It has previously been shown that the CcpN gene is not regulated, which results in a constant concentration of CcpN in the cell under both glycolytic and gluconeogenic conditions. Since CcpN-mediated repression is only required during glycolysis, a ligand is necessary to modulate its activity according to the current metabolic state of the cell.

In vitro transcription assays demonstrated that CcpN is able to exert a semi-specific repression at the three known CcpN-regulated promoters, p_{sr1}, p_{gapB} and p_{pckA}, without any ligand. By contrast, control promoters were not affected by CcpN. This finding corresponds very well to the observation of a rather

**Fig. 6.** Overview of in vitro transcriptions. (a) Summary of the effects of ATP, low pH and ADP on CcpN activity at the sr1, gapB or pckA promoter. Reaction conditions are indicated in the inset. Transcription levels have been normalised at 0 mM CcpN. (b) Summary of the effects of ATP, low pH and ADP on CcpN activity at the sr1, gapB or pckA promoter using CcpN K127A.

**Fig. 7.** EMSA with CcpN under different conditions. EMSAs of double-stranded 89-bp DNA fragments containing the sr1 operators. The DNA was incubated with increasing concentrations of purified CcpN-His6 (CcpN concentration from left to right: 0 nM, 156 nM, 313 nM, 625 nM and 1.25 μM). Specific reaction conditions are denoted under each gel. The autoradiograms of the gels are shown.
significant derepression of \( p_{\text{ockA}} \) in a \( \text{cpN} \) knockout strain.\(^7\) Furthermore, EMSAs performed with \( B. \text{subtilis} \) crude protein extracts revealed a significant amount of bound DNA,\(^9\) which might result from a high CcpN concentration in the cell. Both of these findings provide evidence that the CcpN-regulated promoters are constantly occupied by CcpN and, therefore, partly repressed in \( B. \text{subtilis} \). Transcriptional regulators that are constitutively bound to their operators are not uncommon. One example is the ResD protein from \( B. \text{subtilis} \), which induces the \( \text{yclJK} \) operon under oxygen limitation and constantly occupies a single binding site in the promoter region of this operon.\(^22\) A similar situation can be found with the \( E. \text{coli} \) protein NorR, which acts as a transcriptional activator of the detoxification operon \( \text{norVW} \).\(^23\) However, none of these proteins exert a constant repression or activation upon their regulated operon, as it has been observed in the case of CcpN. It is not unusual that a transcriptional repressor knockout strain shows a slight derepression of its target gene. BzdR from \( Azoarcus \) sp., for example, a repressor of the anaerobic catabolism operon for benzoate, relieves its constitutive repression by a factor of \( \approx 1.3 \) in the \( \text{bzdR} \) knockout strain.\(^24\) Interestingly, an unspecific repression by a factor of 5.4, as exerted by CcpN, is rather peculiar, and its biological relevance remains to be elucidated. 

\[ \beta \]-Galactosidase measurements of \( p_{\text{srs1-lacZ}} \) fusions integrated into different strains with mutations in glycolytic genes revealed that a mutation in the \( \text{gapA} \) gene hardly affects CcpN-mediated repression, while a mutation in the \( \text{pgi} \) gene decreases repression efficiency significantly. This led to the assumption that the wanted ligand is an intermediate in this part of the pathway, namely, fructose-6-phosphate, fructose-1,6-bisphosphate, glyceraldehyde-3-phosphate or dihydroxyacetone phosphate. However, investigation of these substances in \textit{in vitro} transcription did not show a specific CcpN-related effect.

It has previously been shown that CBS domains, of which two are found in CcpN, are able to bind to the adenine part of nucleotides and nucleosides.\(^19\) Surprisingly, ATP did not result in an enhanced CcpN repression in \textit{in vitro} transcription assays with constant CcpN concentration. By contrast, when the CcpN concentration was varied, a small but reproducible effect of ATP was visible: a twofold reduction of the minimal inhibitory concentration of CcpN at all three investigated promoters. This
amount of repression was much smaller than anticipated from the lacZ fusions. This implies that a second factor is required for efficient repression by CcpN. Based on the results obtained with acidic GA3P, a combination of low pH and high ATP concentrations demonstrated that both of these two effectors are required to achieve full CcpN-mediated repression. The dependence on pH is not exceptional, as it is known that a specific pH is required for the correct function of many proteins, among them ion transporters and especially proteases.

It is noteworthy, however, that a pH-sensing function has only been reported for one transcription factor to date, NikR from E. coli, whose sensitivity to nickel is dependent on the current pH in the cell.\textsuperscript{27} In the case of B. subtilis CcpN, the drop in pH in the cell might result from an accumulation of acetate as a final product of the carbon overflow mechanism,\textsuperscript{28} which would fit well into the observed regulation performed by CcpN. If excess glucose is available, the citric acid cycle is shut down, leading to an accumulation of acetate, which is excreted afterwards, and a slight acidification of the cell.\textsuperscript{29,30} This, however, does not explain the repression effect observed in the β-galactosidase measurements with strain SU22. Since none of the investigated substances showed any significant effect in \textit{in vitro} transcription assays, the observations made with this strain might just be an artefact. It could also be possible that one of these substances exerts its effect via a hitherto unknown protein and indeed enhances CcpN-mediated repression \textit{in vivo}.

CD measurements performed with purified CcpN-His\textsubscript{6}, in the presence of ATP or ADP at neutral or acidic pH have strengthened the results of the \textit{in vitro} transcription assays. Obviously, ATP binding to CcpN results in an induced fit mechanism, as significant structural changes occur when increasing concentrations of ATP are present. Such induced fit mechanisms are relatively common for ligand-binding proteins, because they are necessary for their regulatory activity. Examples include the multidrug-binding transcriptional repressor QacR from \textit{Staphylococcus aureus},\textsuperscript{30} a wide range of metabolic enzymes, or the human monoamine oxidase, where structural changes have also been detected by CD spectroscopy.\textsuperscript{31}

**ADP is able to counteract the effect of ATP and HCl**

We demonstrated that ADP is—at equimolar concentrations—able to specifically counteract the effect of ATP. CD measurements reinforced these findings, although the observed effect is not as strong as the effect caused by ATP (Fig. 7). Soga \textit{et al.} have measured the intracellular concentration of metabolites and nucleotides and have shown that, while there is less ADP than ATP in exponentially growing cells, the ATP concentration exceeds the ADP concentration significantly in cells that have entered the stationary phase.\textsuperscript{17,18} In addition to this, it is generally accepted that there is a sharp drop in intracellular ATP concentration upon glucose limitation, ultimately leading to the activation of the RsbW/RsbV system of cellular stress response.\textsuperscript{32,33} Furthermore, it has been demonstrated that CBS domains are able to bind ADP as well as ATP,\textsuperscript{19} which corresponds very well to our findings. It is absolutely feasible that ADP, once its concentration is high enough, replaces ATP in the binding pocket, which leads to structural rearrangements that ultimately result in a relief of CcpN repression. Such counter-regulation can often be observed with enzymes that have to act differently in the presence of certain signal molecules or metabolites, such as aspartate transcarbamoylase from \textit{E. coli} that is stimulated by ATP and inhibited by cytidine triphosphohate (CTP).\textsuperscript{34} However, transcription factors are mostly not counterregulated but have just one ligand that turns them “on” or “off,” for example, BzdR and its ligand benzoyl-CoA from \textit{Azotobacter sp.}\textsuperscript{24} One exception, besides CcpN, is GltC from \textit{B. subtilis}, which is activated by α-ketoglutarate and repressed by glutamate,\textsuperscript{35} making these proteins in this respect a peculiarity. Nonetheless, there are also some differences, as GltC is also regulated by RocG \textit{in vivo},\textsuperscript{36} while YqfL does not influence repression by CcpN.

**CcpN with a mutation in a crucial residue can no longer exert its function**

We have examined a mutation in the CcpN protein in a conserved residue within one of the CBS domains. Aymerich and Declerck (D. Chaix \textit{et al.}, manuscript in preparation) showed that this protein is not active \textit{in vivo} anymore. However, we observed an unspecific CcpN-mediated repression as in the wild-type case. This finding and the fact that the mutant is able to bind to its operator sequence like the wild-type protein imply that the mutation did not affect general DNA binding affinity. Furthermore, Aymerich and Declerck confirmed that this mutant has the same structure as the wild type (D. Chaix \textit{et al.}, manuscript in preparation). Interestingly, this mutant does not respond to ATP. According to Aymerich and Declerck, mutant K127A is no longer able to bind ATP or ADP. Consequently, CcpN mutant K127A is unable to perform specific repression because of the lack of ATP binding ability.

**New working model on CcpN action**

It has been shown that ATP and low pH are specific effectors of CcpN \textit{in vitro} transcription, but they are not able to alter the binding affinity of CcpN to its operator sequence, as revealed by EMSA. Transcriptional regulators that constantly occupy their operator and share this feature with CcpN include NorR from \textit{E. coli} or ResD, part of a two-component system from \textit{B. subtilis}.\textsuperscript{22,23} Such proteins usually operate through alterations in structure, induced by a ligand or another activating signal, such as phosphorylation in the case of ResD. Our CD data clearly demonstrated that at acidic pH, ATP induces significant structural rearrangements in CcpN and, therefore, strongly support this hypothesis. However, what...
is the mechanism of CcpN action? Three main mechanisms can be postulated, and a general model is shown in Fig. 9: CcpN constantly occupies its operator and exerts a certain level of permanent basic repression. It is feasible that CcpN, upon ATP binding, alters its structure in a way that it occupies more space at the promoter region and replaces RNAP, resulting in a classical steric hindrance mechanism, such as that reported for the Fur protein from E. coli. A second possibility would be that ATP-bound CcpN interacts with RNAP. This interaction could influence several phases of transcription initiation. One could imagine that an interaction between CcpN and RNAP inhibits open complex formation, as has been reported for the MerR repressor of E. coli. A third alternative mechanism would be the inhibition of promoter clearance, as shown for protein P4 of phage ϕ29 at the viral A2c promoter. It is interesting to note that, depending on the binding site, CcpN might contact different parts of the RNAP holoenzyme. CcpN bound at site I could contact the C-terminal domain of the α-subunit, while CcpN at site II might form contacts to the sigma factor. Interestingly, it is not yet clear whether two contacts to CcpN at site II might form contacts to the sigma factor. It has been shown that mutations in site I completely abolish CcpN-mediated regulation, this has not been proven for site II. It has been demonstrated that the binding efficiency to two binding sites is orders of magnitude larger than for single sites; hence, it is conceivable that one of the sites is only an auxiliary site whose sole purpose is to increase the affinity for CcpN at this promoter. These hypotheses will be tested in future investigations.

Materials and Methods

Enzymes and chemicals

Chemicals used were of the highest purity available. E. coli RNAP and all chemicals were purchased from Sigma-Aldrich. Taq-polymerase for cloning was purchased from Roche (Germany), and Taq-polymerase for synthesis of in vitro transcription templates was purchased from Solis Biodyne (Estonia).

Strains, media, and growth conditions

B. subtilis strain DB104 (cpp::cat) was used for the preparation of B. subtilis RNAP. B. subtilis strains DB104, QB5331 and SU22 were used for β-galactosidase measurements. The genotypes of these strains can be found in Table 3. TY medium (16 g Bacto tryptone, 10 g yeast extract and 5 g NaCl in 1 l) was used as a complex medium for the purification of RNAP. SP medium [8 g nutrient broth, 0.25 g MgSO₄·7 H₂O, 1 g KCl, 1 ml CaCl₂ (0.5 M), 1 ml MnCl₂ (10 mM) and 2 ml ammonium iron citrate (2.2 mg/ml)] was used as a glucose-free medium for β-galactosidase measurements.

Construction of plasmids for transcriptional lacZ fusions

Plasmid pAC6 (Table 3) was used to insert an EcoRI-BamHI fragment obtained by PCR from chromosomal DNA of B. subtilis DB104 with oligodeoxynucleotides SB 827 and SB 831 (Table 4) to obtain a transcriptional fusion of the sr1 promoter carrying 87 bp upstream of the −35 box and the promoterless lacZ gene. The resulting plasmid pACT87 was integrated into the amyE locus of strains DB104, QB5331 and SU22 and double crossing over was confirmed by streaking the chloramphenicol-resistant transformants on agar with 0.5% starch and subsequent overlay with iodine/potassium–iodide solution.

Construction of a plasmid for inducible ccpN expression

The 2.3-kb BamHI/EcoRI fragment of plasmid pPR1 containing the repR gene was inserted into the pOU71 BamHI/EcoRI vector, yielding plasmid pOUR. A fragment carrying the phleomycin resistance cassette flanked by EcoRI sites was generated by PCR, using primers SB 445 and SB 446 and plasmid pPR1 as template. This fragment was cloned into the pUC19 EcoRI vector, and the sequence was confirmed. The resulting vector was designated pUCP. The EcoRI fragment carrying the phleomycin resistance cassette was then obtained by restriction with EcoRI and inserted into the pOUR EcoRI vector, resulting in plasmid pOURP. Oligonucleotides SB 766 and SB 767 were annealed, yielding a polylinker sequence with 5′ BamHI and 3′ KpnI sticky ends. This fragment was
inserted into the pOURP BamHI/KpnI vector, resulting in plasmid pOU72. A lac repressor fragment was obtained by restriction of plasmid pRS6 with BamHI and XbaI and inserted into the pOU72 BamHI/XbaI vector, resulting in plasmid pOU75. The sequence was confirmed. Plasmid pOU75 was then used for inducible expression of CcpN.

### Overexpression and purification of CcpN

CcpN overexpression and purification with a Ni²⁺-NTA-agarose column were performed as published before. Further purification was performed by streptomycin phosphate precipitation and dialysis against 1× TBE buffer, followed by an anion-exchange chromatography on a HiLoad Q-Sepharose 16/10 column. The protein was dissolved and dialysed against 45 mM Tris/borate buffer, pH 8.3, and applied to the anion-exchange column. Elution of CcpN-His₅ was achieved at about 200 mM NaCl by using a linear elution gradient (2 column volumes) of the same buffer containing 1 M NaCl. The purity and activity of CcpN-His₅ were verified by SDS-polyacrylamide gel electrophoresis (10%) and EMSA.

### Protein concentration determination

The protein concentration of CcpN-His₅ was determined by absorption spectroscopy using a molar extinction coefficient of 5680 l/(mol·cm) at 280 nm according to the method of Gill and von Hippel.

### CD spectroscopy

Far-UV CD measurements were performed on a CD spectropolarimeter J-820 (Jasco). Spectra were recorded at a scan speed of 100 nm/min, at a response time of 2 s and accumulated. The optical path length was 1 mm, and the temperature was set at 20 °C. The protein concentration was 108 µg/ml (4.4 µM) in 45 mM Tris/borate buffer, pH 8.3. The effect of the metabolites on the secondary structure of CcpN-His₅ was determined by titration of the structure of CcpN-His₅ with the metabolites.

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### Table 3. Strains and plasmids used in this study

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### Table 4. Oligonucleotides used in this study

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respective chemical compounds to the protein solution. Spectra were corrected for buffer baseline containing the respective metabolite concentration.

Preparation of templates for in vitro transcription

Double-stranded templates for in vitro transcription were obtained using the corresponding primers (Table 3) in a PCR on chromosomal DNA of B. subtilis DB104. The PCR products were phenolised, extracted with chloroform twice and ethanol-precipitated using 15 mg/l glycogen as carrier. Pellets were washed with 80% EtOH and dissolved in aqua bident. The preparation was analysed on an agarose gel, and subsequently, the DNA concentration was adjusted to 1 μM.

Preparation of B. subtilis RNAP

B. subtilis ccpN knockout strain DB104 (ccpN::caf) was grown in TY medium to an OD600 of 4. Cells were then harvested by centrifugation and sonicated 10 min in sonication buffer [40 mM potassium phosphate, 10 mM ethylenediaminetetraacetic acid, 30 mM NaCl, 10 mM β-mercaptoethanol and 10 mM ethylene glycol bis(β-aminoethyl ether)N,N′-tetraacetic acid]. The supernatant obtained by centrifugation was filtrated through a 100,000 Da molecular mass cutoff Millipore column for 20 min at 6000g to remove smaller proteins and exchange the sonication buffer for RNAP storage buffer (25 mM Tris/HCl, pH 8.4, 1 mM ethylenediaminetetraacetic acid, 7 mM β-mercaptoethanol and 50% glycerol). The preparation was stored at −20 °C.

In vitro transcription

In vitro transcription reactions were performed in a final volume of 10 μl in in vitro transcription buffer (40 mM Tris/acetate, pH 7.5, 10 mM magnesium acetate, 100 mM potassium acetate and 20% glycerol) in the presence of 0.1 mM ATP, CTP and guanosine triphosphate (GTP), 0.01 mM uridine triphosphate (UTP) and 0.01 mM α-[32P]UTP. If indicated, potential ligands were added, followed by 100 nM double-stranded DNA template and 100 nM RNAP. The reaction was gently mixed and incubated for 15 min at 37 °C. One volume of formamide loading dye was added to the reaction, followed by denaturation for 5 min at 90 °C, quick cooling on ice and analysis on a 6% polyacrylamide gel. Electrophoresis was performed at 300 V/25 mA for 50 min. Gels were dried and subjected to PhosphoImaging (Fujix BAS 1000). PC BAS 2.0e software was used for quantification of the bands.

EMSAs

Binding reactions were performed in a final volume of 10 μl in in vitro transcription buffer (see above), 0.05 g/l herring sperm DNA as nonspecific competitor, 1 nM end-labelled DNA fragment and 156 nM to 1.25 μM CcpN-His. All CcpN-His dilutions were made in storage buffer, and the same volume of diluted protein was used in each sample to ensure an equal salt concentration. After incubation at 37 °C for 15 min, the reaction mixtures were separated on 8% native polyacrylamide gels run at room temperature for 1 h at 230 V. Gels were dried and subjected to PhosphoImaging (Fujix BAS 1000).

Acknowledgements

We thank N. Declerck and S. Aymerich for sending us the plasmid for the purification of the CcpN K127A mutant and for inspiring discussion. Furthermore, we thank Jörg Stüke for providing us with the glycolysis mutant strains. In addition, we thank E. Birch-Hirschfeld (Institut für Virologie, Jena) for synthesising the oligodeoxyribonucleotides and Nadine Möbius for helping with the construction of the pSKRlacZ fusions. Plasmid pOU71 was a kind gift from Kenn Gerdes, and we thank Sven Preis for constructing plasmid pGKI. This work was supported by grant BR1352/6-2 from Deutsche Forschungsgemeinschaft (to S.B.). A.L. is financed by a scholarship from the “Fonds der Chemischen Industrie” and a scholarship from the federal state of Thuringia.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2008.05.002

References


5. The transcriptional repressor CcpN from *Bacillus subtilis* uses different repression mechanisms at different promoters.

(Manuskript III)

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Revision eingereicht bei: *Journal of Biological Chemistry* am 03.08.2009
THE TRANSCRIPTIONAL REPRESSOR CCPN FROM *BACILLUS SUBTILIS* USES DIFFERENT REPRESSION MECHANISMS AT DIFFERENT PROMOTERS

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Running head: Repression mechanism of CcpN
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CcpN, a transcriptional repressor from *Bacillus subtilis* that is responsible for the carbon catabolite repression of three genes, has been characterised in detail in the past 4 years. However, nothing is known about the actual repression mechanism so far. Here, we present a detailed study on how CcpN exerts its repression effect at its three known target promoters of the genes *sr1*, *pckA* and *gapB*. Using gel shift assays under non-repressive and repressive conditions, we showed that CcpN and RNA polymerase can bind simultaneously and that CcpN does not prevent RNA polymerase (RNAP) binding to the promoter. Furthermore, we investigated the effect of CcpN on open complex formation and demonstrate that CcpN also does not act at this step of transcription initiation. Investigation of abortive transcript synthesis revealed that CcpN acts differently at the three promoters: At the *sr1* and *pckA* promoter, promoter clearance is impeded by CcpN while synthesis of abortive transcripts is repressed at the *gapB* promoter. Eventually, we demonstrated with far western blots and co-elution experiments that CcpN is able to interact with the RNAP αααα-subunit, which completes the picture of the requirements for the repressive action of CcpN. On the basis of the presented results we propose a new working model for CcpN action.

CcpN, a transcriptional repressor from *B. subtilis* that is responsible for the carbon catabolite repression of at least three genes: *sr1*, encoding a small RNA and *pckA* and *gapB* (1,2), encoding two gluconeogenic enzymes (3,4). Since its discovery in 2005, CcpN has been thoroughly investigated: Binding properties and binding motives were examined, revealing that CcpN possesses two asymmetric binding sites which are bound cooperatively and positioned differently at the three regulated promoters (5): At the *sr1* promoter, binding sites are located upstream of the -35 region and between the -35 and the -10 region, while binding sites cover the -35 as well as the -10 region at the *pckA* promoter. One operator at the *gapB* promoter overlaps the -10 region, the second one is located around +20. ATP and low pH have been identified as signals required for CcpN-mediated repression (6) and the detailed biophysical properties of CcpN-DNA interaction have been reported (7). In addition, it has been shown that CcpN is controlling central carbon fluxes in the metabolism of *B. subtilis* and that the growth defect of CcpN knockout mutants is caused by ATP dissipation via extensive futile cycling (8). It has been demonstrated that a CcpN knockout is able to increase the industrial production of riboflavin in *B. subtilis* by a deregulation of the *gapB* gene (9). However, nothing is known about the actual repression mechanism of CcpN yet.

Initiation of transcription is a stepwise process (10), beginning with binding of RNA polymerase to the promoter and formation of a loose closed complex, which is then rearranged into a tighter closed complex. This is followed by the melting of DNA around the transcriptional start site, called the open complex. RNAP can subsequently form the initiation complex and begin to transcribe the DNA, often producing short abortive transcripts resulting from failed attempts to leave the promoter. Eventually, RNAP escapes the promoter and forms the elongation complex. Transcriptional repressors can act at any of these steps, beginning with steric hindrance of RNAP binding, like the Fur protein of *Escherichia coli* (11) over the inhibition of open complex formation, like *B. subtilis* Spo0A at the *abrB* promoter (12) to the prevention of promoter clearance, as observed with the phage Φ29 protein p4 at the viral A2c promoter (13). Different mechanisms of transcriptional repression have already been reviewed in detail (14).
While steric hindrance of RNA polymerase binding does not involve direct repressor-RNAP contacts, repression of other steps in the transcription initiation process often does. In most of those cases, contacts between a transcriptional repressor and the C-terminal domain of the α-subunit of RNAP are described, as for the p4 protein at the A2c promoter or for the repressor Spx from B. subtilis (13,15). However, interactions with other subunits of RNAP have also been proposed, for example for the Rsd protein of B. subtilis (14,16). A special case of repressors that interact with RNA polymerase subunits are anti-σ-factors. These proteins can sequester free σ-factor and are thus able to influence the expression of whole regulons (18,19).

In this work, we present a detailed analysis of the action of CcpN at all steps of transcription initiation and show that it prevents promoter clearance at the sr1 and pckA promoter, while displaying a rare effect at the gapB promoter: It allows the formation of the open complex, but prevents the synthesis of abortive transcripts. Furthermore, we demonstrate that CcpN is able to interact with the α-subunit of RNAP and probably regulates the sr1 and pckA promoters this way. Eventually, we present a new working model for CcpN-mediated transcriptional repression in regard to the specific operator positions and promoter sequences.

**EXPERIMENTAL PROCEDURES**

*Strains and media used in this study – B. subtilis* strain NIG2001 was used for expression of His-tagged *B. subtilis* RNA polymerase (20) and strain DB104 (21) was used for the preparation of *B. subtilis* protein crude extracts. *E. coli* strain TG1 (pREP4, pQGDR) was used for overexpression and purification of CcpN-His6 and strain BL21 (DE3) (pET5gA) was used for overexpression and purification of His-tagged *B. subtilis* SigA (4,22). All strains were grown in TY medium (16 g Bacto tryptone, 10 g Yeast extract, 5 g NaCl in l 1) with the respective antibiotics.

*Protein purification –* CcpN overexpression and purification with a Ni²⁺-NTA-agarose column and by anion exchange chromatography was performed as published before (6). Expression and purification of Histagged *B. subtilis* RNA polymerase and His-tagged SigA with a Ni²⁺-NTA-agarose column was carried out according to the protocols established by Fujita and Sadaie (20,22).

*Gel shift assays –* Binding reactions were performed in a final volume of 10 µl in either 0.5x TBE and 10 mM MgCl₂ for the formation of closed complexes or in *in vitro* transcription buffer (40 mM Tris/acetate, pH 7.3, 10 mM magnesium acetate, 100 mM potassium acetate and 20 % glycerol) for the formation of open complexes, 0.05 g/l of Heparin were added. After incubation at 37 °C for 15 min, the reaction mixtures were denatured and separated on 5 % native polyacrylamide gels run at room temperature for 1 h at 230 V. Gels were dried and subjected to PhosphorImaging (Fujix BAS 1000).

*Open complex formation assays –* Binding reactions were performed in a final volume of 10 µl in 50 mM sodium-cacodylate buffer (pH 7.3) using 1 nM of an endlabelled DNA fragment. Where indicated, 100 nM of CcpN-His6, 3 µM of RAW TAGS, 0.1 mM of RNAP-His6, 3 mM ATP, HCl to a final pH of 6.5 or 0.1 g/l of Heparin were added. After incubation at 37 °C for 15 min, the reaction mixtures were denatured and separated on 5 % native polyacrylamide gels run at room temperature for 1 h at 230 V. Gels were dried and subjected to PhosphorImaging (Fujix BAS 1000).

*In vitro transcription –* In *in vitro* transcription reactions at the pckA and gapB promoters were performed in a final volume of 20 µl in *in vitro* transcription buffer in the presence of 3 mM ATP, 0.1 mM CTP and GTP, 0.01 mM UTP and 0.011 µM [α-³²P]UTP. For the sr1 promoter, 0.1 mM UTP and 0.011 µM [α-³²P]ATP were used to allow detection of abortive transcripts. Where indicated, HCl to a final pH of 6.5 and CcpN-His6 were added, followed by 100 nM double-stranded DNA template and 100 nM RNAP-
RESULTS

CcpN does not inhibit formation of the closed complex.

Transcriptional repressors can act during a variety of different steps in transcription initiation. To investigate whether CcpN exerts its repression effect by preventing RNA polymerase binding to the promoter, we performed gel shift assays using 89 bp end-labelled double-stranded DNA fragments carrying the srI, pckA, gapB or RNAIII (as a negative control that is unable to bind CcpN) promoters (Figure 1). Purified CcpN-His$_6$ and purified His-tagged B. subtilis RNA polymerase alone and together were incubated with the labelled DNA fragment and complex formation was analysed on native polyacrylamide gels. The presence of CcpN or RNA polymerase alone resulted in a single band corresponding to the respective protein-DNA-complex at all three promoters. When both proteins were present, an additional band was visible at all three promoters, emerging from a complex of DNA, CcpN and RNA polymerase. As expected, the control promoter of RNAIII showed only a single band corresponding to an RNAP-DNA complex, but no CcpN-DNA-complex. All experiments were performed under non-repressive (0 mM ATP, pH 7.3) and under repressive conditions (3 mM ATP, pH 6.5) to assay if CcpN is able to prevent RNA polymerase binding to the promoter sequence. For analysis under repressive conditions, both ATP and low pH were also present in the gel and in the running buffer to ensure that the conditions did not change during electrophoresis. At all three promoters, the intensity of the band representing the CcpN-RNAP-DNA complex did not change in intensity when comparing non-repressive with repressive conditions, indicating that CcpN is not able to prevent the formation of the closed complex.

CcpN does not inhibit open complex formation.

The next step in transcription initiation is the formation of the open complex, involving melting of the DNA at the promoter region. In order to detect formation of an open complex, a double stranded DNA fragment was probed for the presence of single-stranded regions under different conditions using DEPC (Figure 2), which is known to react preferentially with single-stranded regions in B-form DNA (23). Usually KMnO$_4$ is used for detection of single-
stranded regions, but did not work under our buffer conditions. Therefore, DEPC was used, although it has the disadvantage of producing weaker signals at stacked adenosine residues. As shown in Figure 2, signals emerged at all three promoters upon addition of RNA polymerase that were not present in the negative control, where only DEPC was added. These signals persisted in the presence of CcpN (non-repressive conditions) as well as in the presence of CcpN, ATP and low pH (repressive conditions) at all investigated promoters. Thus, one can conclude that CcpN is not able to prevent formation of the open complex at any of the three promoters. To corroborate these findings, another assay for open complex formation using Heparin as a probe has been performed. Again, CcpN was not able to prevent the formation of open complexes at repressive or non-repressive conditions at any of the investigated promoters (Figure 2, bottom panels).

**CcpN acts differently at the three promoters.**

Since formation of the open complex is not impeded by CcpN at any promoter, it can either prevent the synthesis of abortive transcripts or promoter clearance. To investigate this issue, in vitro transcription reactions under non-repressive and repressive conditions were performed and analysed on two different denaturing polyacrylamide gels: 6 % gels were used to detect full-length transcripts while 23 % gels were used to detect abortive transcripts (Figure 3). Since there is no uridine within the first 11 bases of the sr1 transcript, [α-32P]ATP instead of UTP was used for labelling. This resulted in very faint bands for both the full-length and the abortive transcripts, because all in vitro transcription reactions were performed in the presence of 3 mM ATP necessary to observe the repressive effect of CcpN. To ensure that the observed abortive transcripts are produced by the analysed promoters rather than non-promoter sites on the template, templates with mutated promoters were investigated (Figure 3). Indeed, certain transcripts within the expected size of 3-11 nt are no longer produced from the mutated fragments, indicating that they emerge from the investigated promoters. At all three promoters, formation of full-length or abortive transcripts was not influenced in the presence of CcpN or low pH alone. Figure 3A shows that abortive transcripts are produced at the sr1 promoter in all four lanes, even under repressive conditions, while synthesis of the full-length transcript is significantly repressed in the presence of CcpN, ATP and low pH. At the pckA promoter, most of the abortive transcripts are still produced during CcpN-mediated repression, however, the smallest two transcripts are lost. Nevertheless, Figure 3B clearly shows that abortive transcription in general is not affected by CcpN. A completely different picture can be found at the gapB promoter (Figure 3C). Here, bands corresponding to abortive transcripts are hardly or not at all detectable under repressive conditions. Thus, one can conclude that CcpN acts at the sr1 and pckA promoters by preventing RNA polymerase from leaving the promoter and proceeding with transcription, while still allowing the production of short abortive transcripts. At the gapB promoter, however, CcpN impedes transcription initiation itself, resulting in the inability to produce abortive transcripts.

**CcpN is able to interact with RNA polymerase**

Since CcpN is able to prevent RNA polymerase from leaving the promoter, we wanted to find out whether this is due to a direct interaction. To this end, we purified the RNA polymerase α-subunit (RpoA) as well as the B. subtilis major σ-factor SigA. Figure 4A shows the two proteins, along with BSA and purified CcpN. While SigA is apparently pure, the alpha subunit contains some impurities, although in a much lower concentration than the protein itself. Two gels with identical protein samples have subsequently been subjected to far western blotting to analyse possible interactions between CcpN and these proteins (Figure 4B). The left panel shows the control blot that was only incubated with primary (anti-CcpN) and secondary antibody. As expected, CcpN itself produced a very strong signal, indicating that the antibodies work as intended. However, there are also two signals in the lane with the RpoA preparation: A very intensive signal corresponding to the largest impurity, indicating extensive antibody cross-reaction and a weak signal at the 27 kDa impurity. The RpoA band itself did not produce a signal, demonstrating that the anti-CcpN-antibody did not bind to it unspecifically. Furthermore, there were no antibody cross reactions with either SigA or BSA. The right panel shows the experiment itself, where the blot has been incubated with CcpN before the application of the first
antibody. Strikingly, a band emerges that corresponds exactly to the 39 kDa band comprised of RpoA, indicating that CcpN is able to specifically interact with the RNA polymerase α-subunit. Neither SigA nor BSA showed any interaction with CcpN at all. To corroborate these findings, we investigated whether CcpN can be co-eluted with an α-subunit preparation. To this end, a crude extract of *B. subtilis* DB104 was incubated with RpoA-His6 and subsequently purified using a Ni²⁺-NTA-agarose column. As controls, RpoA-His6 alone, the crude extract alone as well a crude extract preincubated with SigA-His6 were purified in the same manner. Figure 4C shows the results of these experiments. It can be clearly seen that only in the case where the crude extract was preincubated with RpoA, a new band emerges that corresponds to native CcpN. As expected, this band runs marginally faster than the purified CcpN due to the lack of the His-Tag used for CcpN purification. Taken these results and the far western blot together, one can conclude that CcpN is able to specifically interact with the α-subunit of RNA polymerase.

**DISCUSSION**

**Repression mechanism of CcpN**

Here, we report the elucidation of the repression mechanism employed by the transcriptional repressor CcpN from *B. subtilis*. Gel shift assays demonstrated that CcpN does not prevent RNA polymerase binding and that both proteins can bind simultaneously to the promoter. Interestingly, CcpN and RNA polymerase, although able to bind simultaneously, appear to compete for binding to the used DNA fragments. Figure 1 clearly shows that the bands for all three complexes are significantly weaker when both proteins are present than the complexes where only one of the proteins is present. Since CcpN and RNA polymerase concentrations have been chosen to reflect their actual concentrations in vivo (unpublished observation, 24), it is conceivable that there is also a competition between these two proteins for promoter binding within the cell. This finding would also explain the observations made by Servant et al., who reported a significant derepression of the *pckA* and *gapB* promoters in a *ccpN* knockout mutant, even under gluconeogenic conditions where CcpN is not active (3), a feature that was also reported for other transcriptional repressors, although not to such a huge extent (25).

Repressors that bind simultaneously with RNA polymerase, either at overlapping or at different sites, often repress transcription by preventing melting of DNA at the transcriptional start site, i.e. formation of the open complex. Such transcription factors are for example *E. coli* MerR at the *merT* promoter (26,27), which binds together with RNAP at opposite sites of the DNA helix, or the KorB protein of broad host range plasmid RK2 (28), whose binding sites do not overlap those of RNAP. CcpN features both versions of operator sites, some overlap with RNAP binding sites whereas some do not (5). However, open complex formation assays clearly ruled out the possibility that CcpN acts by preventing DNA melting at any of the promoters.

The inhibition of the synthesis of abortive transcripts, as observed by us at the *gapB* promoter, is a case rarely reported in literature. The H-NS protein at the *rrnB* P1 promoter or the FIS protein at the *gyrB* promoter are two examples for this kind of repression (29,30). For H-NS, a binding pattern similar to CcpN has been reported, where the operator overlaps the RNAP binding site. H-NS is then able to alter the DNA structure at this position, allowing the formation of open complexes, but preventing subsequent transcription. A similar mode of action is conceivable for CcpN at the *gapB* promoter. DNase I footprints have revealed the appearance of several hypersensitive sites upon CcpN binding at this promoter, which is usually a good indication for structural alterations of the DNA (3,4). At the *sr1* and *pckA* promoter, however, abortive transcripts are readily formed, but escape of RNA polymerase from the promoter is inhibited. Prevention of promoter clearance is usually mediated by one of two different ways: A repressor can bind downstream of RNAP and simply create a roadblock before a stable elongation complex can be formed. This has for example been shown for CcpA-mediated regulation of the *treP* gene in *B. subtilis*, and even as a prove of principle with an artificial construct using the Lac repressor (31,32). Regarding the operator positions at the *sr1* and *pckA* promoters, this mechanism appears to be highly unlikely, which favours the second alternative possibility: An interaction between the repressor molecule and parts of the RNA
polymerase. It is known that the polymerase can be stalled at promoters with close-to-consensus sequences, resulting from extremely tight binding that subsequently makes promoter clearance very difficult (33). Transcriptional repressors, which usually bind their operator sequences with high affinity, can mimic the aforementioned effect by binding RNAP and keeping it in place. Examples for this mechanism include the phage Φ29 protein p4 at the phage A2c promoter (34) and the Gal repressor (35).

CcpN interacts with the RNAP-α-subunit

With respect to our finding that CcpN is able to specifically interact with the RNA polymerase α-subunit, we conclude that CcpN acts as a repressor at the sr1 and pckA promoters by keeping RNAP in place through the aforementioned interaction. There are various reports about the α-subunit, and especially the C-terminal domain, being an interaction interface for transcriptional repressors, as mentioned above. However, interactions with the α-subunit have also been reported for activators, like CcpA at the ackA promoter (36,37) or SoxS during oxidative stress conditions (38). Considering the binding site position of CcpN at the sr1 and pckA promoters, an interaction with the α-subunit appears very conceivable. It has been shown that up elements in B. subtilis have a slightly broader tolerance regarding location than in E. coli (39,40), reaching approximately from -40 to -66, which would position the α-C-terminal domain to be able to interact with CcpN at these promoters.

At the gapB promoter, however, an interaction between the α-subunit can be excluded, since both operator sites are at far downstream to allow any contact between the two proteins. Two possibilities are conceivable: CcpN exerts its action here: Either CcpN alters the DNA structure as mentioned above, or it interacts with an RNAP subunit other than the α-subunit or the σ-factor, since the first one cannot be contacted and no interaction has been detected with the latter. Reports of transcription factors that interact with e.g. the β-subunit are quite uncommon. One of these examples is the AsiA protein from bacteriophage T4 (41), another being the Rsd protein of E. coli (42), both of which have been shown to be able to interact with the core RNA polymerase. If CcpN actually interacts with parts of the RNAP other than the α-subunit needs to be experimentally determined. However, the relatively small size of CcpN, leaving not much space for extensive interaction surfaces and the fact that DNA structure is altered upon CcpN binding seem to favour the possibility of repression by DNA-structure rearrangements.

The example of CcpN shows that one single repressor can exert repression in very different ways, depending on how its operators are positioned relative to the RNA polymerase binding sites. Varying binding site distribution is quite common, found e.g. in the case of CytR from E. coli (43) and many more. Interestingly, cases where variations in operator positioning result in different repression mechanisms have not been frequently reported in literature. However, this is mostly because the actual repression mechanism for these proteins has not been elucidated. A well documented example where operator site positions have an impact on the repression mechanism is cre element positioning, allowing CcpA to exert a broad range of repression or even activation mechanisms on its targets (17).

Taking all results together, a quite clear picture of the repression mechanism of CcpN can be established where CcpN and the α-subunits are in a spatial position that allows interaction and subsequent promoter arrest at the sr1 and pckA promoters, but not at the gapB promoter. Here, repression by modification of the DNA structure appears to be a probable alternative.

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We are very grateful to M. Salas for the gift of native purified *B. subtilis* RNA polymerase as well as for the strain for the overproduction of His-tagged α-subunit. Furthermore, we would like to thank M. Fujita, who kindly sent us the strain for overproduction of His-tagged σ-factor. This work was supported by grant BR1552/6-3 from Deutsche Forschungsgemeinschaft (to S. B.). A. L. was financed by a scholarship from the federal state of Thuringia and by the Deutsche Forschungsgemeinschaft.

**FOOTNOTES**

**FIGURE LEGENDS**

Fig. 1. EMSAs with His-tagged CcpN and RNA polymerase at the *sr1* (A), *pckA* (B), *gapB* (C) and *RNAIII* (D) promoters. The presence or absence of 3 µM CcpN-His$_5$ and 3 µM RNAP-His$_6$ is indicated above each lane. Experiments were performed under non-repressing (pH 7.0; 0 mM ATP) or repressing (pH 6.5; 3 mM ATP) conditions. Autoradiograms of the gels are shown. F: free DNA, CD: CcpN-DNA-complex, RD: RNAP-DNA-complex, CRD: CcpN-RNAP-DNA-complex.

Fig. 2. Open complex formation assay at the *sr1* (A), *pckA* (B) and *gapB* (C) promoters. Probing with DEPC is shown at the top while the corresponding Heparin-probing is shown below. For DEPC-probing, DEPC (10 %), RNAP (100 nM), CcpN-His$_5$ (100 nM), were added where indicated. Bands showing the presence of single stranded DNA regions and therewith open complexes are indicated by arrows. G: G>A sequencing reaction, C: C+T sequencing reaction. Positions of +1, the -10 and -35 box are indicated. Please note that the noncoding strand was used for *sr1* and *pckA*, while the coding strand was used for *gapB*. For Heparin-probing, Heparin (0.1 g/l) CcpN-His$_5$ (3 µM), RNAP-His$_6$ (3 µM) ATP (3 mM) or HCl (to a final pH of 6.5) were added where indicated. F: free DNA, CD: CcpN-DNA-complex, RD: RNAP-DNA-complex, CRD: CcpN-RNAP-DNA-complex. Autoradiograms of the gels are shown.

Fig. 3. *In vitro* transcription and detection of abortive transcripts at the *sr1* (A), *pckA* (B) and *gapB* (C) promoters. Transcription was performed in *in vitro* transcription buffer (see experimental procedures) using 100 nM DNA template and 100 nM His-tagged *B. subtilis* RNA polymerase. 300 nM CcpN-His$_5$ was added or pH was lowered where indicated. Half of each reaction was separated on either a 6 % denaturing polyacrylamide gel to detect the full length transcripts, indicated by an arrow or on a 23 % denaturing polyacrylamide gel to detect abortive transcripts, indicated by a bracket. Control experiments to the right of each panel show which of the abortive transcripts are produced by the investigated promoters. WT: wild-type promoter, MUT: mutated promoter, where the -10 regions have been replaced by the sequence GCCGAT (*sr1*) or GCCGCT (*pckA* and *gapB*). The estimated size of the abortive transcripts on each gel is indicated by arrows. Autoradiograms of the gels are shown.

Fig. 4. CcpN-RpoA interaction studies. The corresponding molecular weights of the marker bands are indicated beside the marker lanes. (A) 15.5 % SDS-polyacrylamide gel of different purified proteins. MW: molecular weight marker; σ: purified SigA-His$_6$; α: purified RpoA-His$_6$; BSA: bovine serum albumin; CcpN: purified CcpN-His$_5$. 1 µg of each protein was loaded into each lane. (B) Far western blot of the protein gel shown in (A). Equal amounts of protein were loaded into lanes 1-4 and 5-8, respectively. Proteins were renatured after blotting by washing with SDS-free PBS. Lanes 1-4 are control lanes and were just blocked, incubated with rabbit-anti-CcpN antibody and subsequently with horseradish peroxidase coupled anti-rabbit antibody. Lanes 5-8 are the sample lanes and were treated like lanes 1-5, but were incubated with 200 nM CcpN-His$_5$ after blocking and before incubation with anti-CcpN antibody. The blots were developed using horseradish peroxidase catalysed conversion of diaminobenzidine. PC-BAS 2.08e software was used for quantification. (C) Co-elution of RpoA-His$_6$ and CcpN. The lanes were loaded as follows: CE+α: RpoA-His$_6$ preincubated with *B. subtilis* DB104 protein crude extract (see experimental procedures) and subsequently purified using a Ni$^{2+}$-NTA-agarose column; α: RpoA-His$_6$ without preincubation with *B. subtilis* DB104 protein crude extract; CE: *B. subtilis* DB104 protein crude extract, purified; CE+σ: SigA-His$_6$ preincubated with *B. subtilis* DB104 protein crude extract and subsequently purified; CcpN: purified CcpN-His$_5$. Equal amounts of eluate were loaded into each lane.
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**Figure 1 Licht et al.**
CcpN
ATP/H

DEPC
RNAP
CcpN
ATP/H
G C
- - - + +

-35

-10

+1>

Heparin
RNAP
CcpN
ATP/H

CRD
CD
F

Figure 2 Licht et al.
Figure 3 Licht et al.
Figure 4 Licht et al.
6. Search for additional targets of the transcriptional regulator CcpN from *Bacillus subtilis*.

(Rudiments IV)

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Akzeptiert bei: *FEMS Microbiology Letters* am 31.07.2009
SEARCH FOR ADDITIONAL TARGETS OF THE TRANSCRIPTIONAL REGULATOR CCPN FROM BACILLUS SUBTILIS

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Abstract

Transcriptional repressor CcpN from B. subtilis mediates the CcpA-independent catabolite repression of three genes, sr1 encoding a small regulatory RNA, and two gluconeogenesis genes, gapB and pckA. The intracellular concentration of CcpN was determined to be around 4000 molecules per cell. The B. subtilis genome was scanned for potential new CcpN target genes, out of which three showed CcpN binding activity in their upstream region. EMSAs demonstrated that the promoter regions of two putative targets, thyB encoding thymidylate synthase B and yhaM encoding a 5'-3' exoribonuclease, bound CcpN with significant affinity. A detailed contact probing of CcpN-DNA interactions revealed an interesting new binding pattern at the thyB promoter, where the whole promoter appears to be contacted by CcpN. Using lacZ-reporter gene fusions and in vitro transcription assays, the thyB promoter was investigated for a regulatory effect of CcpN. Surprisingly, CcpN does not repress transcription at this promoter, but instead acts as an activator. Alignments of the thyB promoters of different Gram-positive bacteria encoding CcpN revealed CcpN consensus binding sites in a significant number of them. Our data show that a bioinformatics-based approach combined with in vivo and in vitro experiments can be used to identify new targets of transcriptional regulators.

Keywords: CcpN / transcriptional regulator / carbon catabolite repression / footprinting / alignment

Introduction

Catabolite repression is an important regulatory aspect in a variety of bacteria, among them Bacillus subtilis (Chambliss, 1993; Steinmetz, 1993). In B. subtilis, this process is mediated primarily by the combined action of CcpA and HPr-Ser46-P by forming a transcriptional regulator upon interaction (Chambliss, 1993; Stülke & Hillen, 2000). However, carbon catabolite repression of at least three known genes, gapB, pckA and sr1, is mediated by the transcriptional repressor CcpN (Licht et al., 2005; Servant et al., 2005). CcpN binds cooperatively to two distinct binding sites at each of these promoters and has recently been shown to require ATP and a slightly acidic pH for the exertion of its repression effect, while ADP was able to counteract the ATP-mediated repression (Licht & Brantl, 2006; Licht et al., 2008). The repression mechanism of CcpN has not been elucidated so far.

Bacterial transcriptional regulators can act as pure activators, e.g. MalT, the activator of maltose metabolic genes, or PhoB, an activator controlling phosphate uptake in Escherichia coli (Schlegel et al., 2002; Yamada et al., 1989), as pure repressors repressors like the Arg or Lac repressor from E. coli (Maas, 1994; Lewis, 2005) or as dual regulators acting either as activator or repressor as e.g. the global regulators CcpA and CodY from B. subtilis (Henkin, 1996; Sonenshein, 2005).

The number of genes regulated by a transcription factor varies widely: Some of them regulate only one single gene or operon, like the Lac repressor. These regulators are often present in rather low intracellular concentrations, e.g. 10-20 tetramers in case of the Lac repressor (Lin &Riggs, 1975; von Hippel et al., 1974) Others, like MalT, regulate a small set of genes or operons (Schlegel et al., 2002), while others regulate a significant amount of genes as e.g. NarL that is - together with 6 other regulators - responsible for the control of 50 % of all genes in E. coli (Stewart, 1994).

Hitherto, CcpN could be characterised as a pure repressor controlling a small set of genes,
and although efforts have been made to identify more targets of CcpN by microarray analysis and comparative transcriptome analysis, these attempts had remained unsuccessful (Servant et al., 2005, Tännler et al., 2008).

The aim of the present work was to identify potential new targets for CcpN. One promising new target, the thyB gene, has been investigated in detail. We demonstrate that CcpN is able to modestly activate transcription from the thyB promoter. Our results show that bioinformatics in combination with experimental methods is a powerful tool to identify new targets of transcriptional regulators.

**Materials and Methods**

**Enzymes and chemicals**

Chemicals used were of the highest purity available. *E. coli* RNA polymerase and all chemicals were purchased from Sigma-Aldrich™. Taq-polymerases were purchased from Roche (Germany) and Solis Biodyne (Estonia).

**Strains, media and growth conditions**

*B. subtilis* strains DB104 (Kawamura & Doi, 1984) and DB104 (ccpN::cat) (Licht et al., 2005) were used. TY medium (Licht et al., 2005) served as complex medium. SP medium (Preis et al., in press) served as glucose-free medium. Strain NIG2001 was used for expression of His-tagged *B. subtilis* RNA polymerase (Fujita & Sadaie, 1998b). *E. coli* strain DH5α was used for cloning and strain BL21 (DE3) (pETSigA) for overexpression and purification of His-tagged *B. subtilis* SigA (Fujita & Sadaie, 1998a).

**Overexpression and purification of proteins**

CcpN overexpression and purification were performed as published before (Licht et al., 2008). Expression and purification of His-tagged *B. subtilis* RNA polymerase and His-tagged SigA were carried out as described (Fujita & Sadaie, 1998a, 1998b).

**Determination of the intracellular concentration of CcpN in *B. subtilis***

The intracellular concentration of CcpN was determined following the procedure described for CopR (Steinmetzer et al., 1998) except that the Western blot was developed with diaminobenzidine.

**EMSAs and footprinting experiments**

EMSAs, methylation and potassium permanganate interference footprinting were performed as described (Licht & Brantl, 2006). DNase I footprinting was performed as described (Licht et al., 2005).

**Construction of plasmids for transcriptional lacZ fusions and measurements of β-galactosidase activities**

Plasmid pAC6 was used to insert an *EcoRI-BamHI* fragment obtained by PCR from chromosomal DNA of *B. subtilis* with oligodeoxyribonucleotides SB1069 (Table S1) and SB1070 yielding plasmid pTHY1. For plasmid pATM2, oligodeoxyribonucleotides SB1268 and SB1069 as well as SB1267 and SB1070 were used on chromosomal DNA of *B. subtilis* DB104 as template to create fragments MUT2up and MUT2down, respectively. A second PCR using these fragments and oligodeoxyribonucleotides SB1069 and SB1070 resulted in fragment MUT2, carrying the thyB promoter and a mutated CcpN operator site, which was inserted as an *EcoRI-BamHI* fragment into plasmid pAC6. Integration of the plasmids into the amyE locus and measurements of β-galactosidase activities were performed as described previously (Brantl, 1994).

**In vitro transcription**

*In vitro* transcription reactions were performed in a final volume of 10 µl in *in vitro* transcription buffer (40 mM Tris/acetate, pH 7.5, 10 mM magnesium acetate, 100 mM potassium acetate and 20% glycerol) in the presence of 1 mM GTP, 0.1 mM ATP, 0.1 mM CTP, 0.01 mM UTP and 0.011 µM [α-32P]UTP with templates generated as described (Licht et al., 2008). If appropriate, effectors were added, followed by the addition of 100 nM of double-stranded DNA template and 50 nM of His-tagged *B. subtilis* RNA polymerase and 50 nM SigA-His6 and incubation for 30 min at 37 °C. Samples were treated with formamide loading dye and separated on a 6 % denaturing polyacrylamide gel at 300 V/25 mA for 50 min. Dried gels were subjected to PhosphorImaging as above.

**Results and discussion**

*CcpN is an abundant protein in *B. subtilis***

To determine the intracellular concentration of CcpN, protein crude extracts from *B.
subtilis DB104 in TY – together with purified CcpN of known concentration – were analysed by western blotting as described in Materials and Methods (Fig. 1). Since CcpN is constitutively expressed in log and stationary phase, cultures from OD_{560} = 4.0 were used. The amount of CcpN was calculated to be 4000 ± 600 molecules per cell. Taking into consideration a B. subtilis cell volume of 1 \times 10^{15} \text{l} (Abril et al., 1997), the intracellular concentration of CcpN is approximately 6.6 \mu M. Intracellular amounts of transcriptional regulators vary from few molecules, e.g. 10-20 in case of the Lac repressor from E. coli (Lin & Riggs, 1975), to approximately 15000 like in the case of CopR, a transcriptional repressor regulating the copy number of streptococcal plasmid pIP501 (Steinmetzer et al., 1998). The pleiotropic regulators CcpA or CodY from B. subtilis are present in amounts resembling the one of CcpN, namely 3000 (Miwa et al., 1994) and \approx 2500 (A. L. Sonenshein, personal communication) molecules per cell, respectively. Intracellular repressor concentrations – in the case of chromosomally encoded repressors – appear to correlate at least partially with the amount of genes they regulate. The Lac repressor, present in very low concentration, regulates only one operon, while CcpA and CodY are involved in the direct regulation of 100 and 25 genes or operons, respectively (Sonenshein, 2007; Sonnenshein, 2005). Therefore, we wanted to find out if CcpN might regulate more than the three known genes gapB, pckA and sr1.

A database search reveals 291 potential CcpN targets

Therefore, the genome of B. subtilis was searched for possible CcpN binding sites using the SubtiList Web Server (http://genolist.pasteur.fr/SubtiList/) and a slightly revised version of the CcpN consensus sequence (TRTGHYATAYW) reflecting naturally occurring binding sites as well as binding sites found by EMSA (Licht et al., 2005, Servant et al., 2005). Additionally, one mismatch in the consensus sequence was allowed and only sequences within −100 bp or +20 bp relative to the translational start site were considered, since the known CcpN operators are located within this range (Servant et al., 2005) and the location of the promoters of many genes is still unknown. 291 putative CcpN binding sites were found, among them 22 that perfectly matched the consensus sequence.

EMSA suggests at least three additional targets of CcpN

Out of all potential CcpN targets found, those encoding proteins involved in carbon catabolism or those whose CcpN operator sequence was matching the consensus binding site were selected for further investigation. EMSAs were performed using fragments carrying the putative CcpN operator in parallel with a fragment carrying the consensus binding site of the sr1 promoter (Fig. 2). Of 20 investigated operators, only three were bound by CcpN: thyB, gcaD and yhaM. However, binding was less efficient than for the sr1 operator. Apparent K_D values were 770 nM for thyB, 2.9 \mu M for yhaM and 3.4 \mu M for gcaD, compared to 420 nM for the sr1 single site K_D value. Although the thyB operator shows one mismatch to the consensus sequence, its K_D value is still significantly higher than the K_D for yhaM, which is almost a perfect consensus sequence.

The thyB gene encodes the minor thymidylate synthase of B. subtilis, contributing to only 5% of thymidylate synthesis (Neuhard et al., 1978). Interestingly, the main thymidylate synthase of B. subtilis is closely related to thymidylate synthases encoded by phages, while thyB resembles the thymidylate synthases found in other bacteria (Tam & Borriss, 1998). The yhaM gene codes for a 5'–3' exoribonuclease (Oussenko et al., 2002) and gcaD encodes UDP-N-acetylgalactosamine pyrophosphorylase involved in cell-wall buildup (Hove-Jensen, 1992).

Since the K_D values for CcpN in the putative gcaD and yhaM operators indicated a very weak binding, we focused on thyB in further experiments.

DNase I footprinting reveals one large binding site for thyB

All previously investigated CcpN operators have two binding sites, one closely resembling the consensus binding site and one vastly differing from it (Licht & Brantl, 2006; Servant et al., 2005). To determine binding sites at the thyB promoter, DNase I protection footprinting was performed (Fig. 3a). A situation resembling the pckA promoter was found, with only one long stretch of protected
bases that includes both the –10 and the –35 region. As in pckA, the consensus binding site overlapped with the –10 region. The regions protected by CcpN are summarised in Figure 3a.

At the sr1, pckA and gapB promoters, there is one strong and one weak binding site, but these differences are overcome by CcpN binding cooperatively to the two sites, so that both sites are bound with the same efficiency when they are present on one DNA fragment and equally well protected from DNase I (Licht & Brantl, 2005). Interestingly, protection from DNase I was not constant within the protected region at the thyB promoter.

To verify that CcpN binds specifically at the thyB promoter, a thyB fragment carrying a mutation in the CcpN consensus binding site was subjected to DNase I footprinting. Indeed, no footprint was obtained (Fig. 3a).

Chemical interference footprinting identifies bases contacted by CcpN at the thyB promoter

Chemical interference footprinting experiments were performed to determine protein-DNA contacts at a higher resolution. Since neither contacts to C residues nor to the sugar-phosphate backbone played a significant role in the previously studied CcpN-operator interactions (Licht & Brantl, 2006) only methylation and KMnO₄ interference footprints were performed to detect G and A or T residues contacted by CcpN, respectively.

The thyB promoter shows a very unusual contact distribution compared with the previously investigated CcpN operators (Fig. 3b+c). The closest contacts are located within the consensus sequence, but the other contacts are almost evenly distributed over the remaining region protected in DNase I footprinting. A similar DNase I protection pattern was found at the pckA promoter, but chemical footprinting showed clearly two distinct binding sites, located two helical turns apart (Licht & Brantl, 2006). At the thyB promoter, three binding sites are present in total, one strong and two weaker ones. Since in DNase I footprints primarily the consensus sequence was protected, the other two binding sites might be auxiliary sites serving to guide CcpN to its main binding site – a feature that can also be observed with the Lac repressor (Oehler et al., 1990). At the thyB promoter, contacts to T’s are more prominent than contacts to G’s and A’s, whereas at the sr1, gapB and pckA promoters Gs were the bases forming the closest contacts with CcpN. Interestingly, the upstream binding site seems to rely mainly on contacts to A’s whose modification occurs exclusively in the minor groove indicating that CcpN is able to access this much narrower DNA groove. Minor groove binding is often found in the form of ‘indirect readout’, like in CRP or HU of E. coli (Lindemose et al., 2008, Swinger & Rice, 2007) and does not provide much binding specificity due to the low information content. However, it greatly increases binding stability. ComK, the B. subtilis competence regulator, was found to specifically bind its operator site through contacts in the minor groove, which leads to a novel potential activation mechanism (Smits et al., 2007). It is possible that CcpN exerts the same mechanism at the thyB promoter.

A comparison of previously investigated CcpN operators and operator at the thyB promoter can be found in table S2.

Reporter gene assays and in vitro transcription demonstrate that CcpN acts as an activator at the thyB promoter

To assay the in vivo relevance of CcpN binding to the thyB promoter, transcriptional thyB-lacZ fusions were integrated into the chromosome and β-galactosidase activities measured. Strains were grown in glucose-free SP medium and glucose added where appropriate. The comparison between DB104 wild-type and isogenic ccpN knockout strain allowed for detection of an influence of CcpN. The results showed a small but significant and reproducible effect of CcpN (Fig. 4a). Whereas transcription is increased by a factor of 1.5 upon glucose addition in the wild-type strain, this effect cannot be observed in the ccpN knockout strain, where transcription levels in the absence and presence of glucose are nearly identical. To substantiate these results, a lacZ-fusion with a thyB promoter carrying a mutation that prevents CcpN-operator-interactions (see Fig. 3a) was constructed and analysed as above. The site and type of mutation was chosen based on previously performed EMSAs (Licht et al., 2005), where the mutated base has been shown to be invariant. This construct did not respond to glucose, indicating that CcpN truly regulates the thyB promoter (Fig. 4a). This finding is particularly striking, since CcpN has only been shown to work as a repressor under glycolytic conditions.
conditions (Licht et al., 2005, Servant et al., 2005), but here, it acts as an activator. A statistical significance test confirmed the difference in activation between wild-type and knockout strain with a confidence of > 98%. Compared to the huge effects CcpN exerts as a repressor, the relatively small effect observed here might be due to the significantly greater $K_D$ value observed for the thyB promoter. Beside CcpN, there are few other examples of DNA-binding proteins that use an identical consensus sequence for both activation and repression. Two prominent examples are the global regulators CodY and CcpA from B. subtilis (Henkin, 1996; Sonenshein, 2005), the former clearly resembling the case of CcpN, whereas the latter requires HPr as a co-regulator and acts as activator or repressor depending on operator positions.

To corroborate these findings, in vitro transcription experiments with B. subtilis RNA polymerase were performed (Fig. 4b). This assay clearly showed that CcpN is able to activate thyB transcription under conditions of low pH and high ATP concentration, conditions found to be required for CcpN at the three known target promoters. This is in good agreement with the results of the β-galactosidase measurements, showing that CcpN is able to activate the transcription of thyB both in vivo and in vitro. It remains unclear how CcpN is able to act as an activator upon binding to the -10 region, but other studies have shown the same effects for B. subtilis CodY at the ackA promoter and the E. coli MerR protein (Shivers et al., 2006; O’Halloran et al., 1989).

Regarding the physiological relevance of CcpN-mediated activation of thyB transcription, one could hypothesise that more thymidylate is needed during glycolysis, which usually comes along with excellent growth conditions and increased growth rate. ThyB contributes to only about 5% of TSase activity in B. subtilis (Neuhard et al., 1978), and an activity increase of 50% does not seem relevant in the background of ThyA. However, ThyB might be the “original” TSase of B. subtilis’ ancestors, and while its activity has been almost lost, the regulatory mechanism is still intact. The results presented here might also indicate that ThyB is involved in the formation of pyrimidine nucleotide activated sugars during glycolysis and therefore upregulated independently of ThyA. Since it is not excluded that CcpN might regulate thymidylate synthetase genes in other Gram-positive bacteria that possess – in contrast to B. subtilis - only one thy gene, an alignment of CcpN binding sites at thyB promoters in related Gram-positive species was performed.

Alignments show potential CcpN binding sites at thyB promoters in related Gram-positive species

To investigate if CcpN operator sites are located upstream of thyB genes in these species, all bacteria encoding CcpN homologues were scanned for thyB related genes using BLAST (Zhang et al., 2000). Species with the highest thyB homology compared to B. subtilis were subsequently searched for CcpN operator sites upstream of the thyB start codon, since the transcriptional start sites are not known in most of these organisms. Figure 5 shows the alignments. Interestingly, in B. amyloliquefaciens, B. anthracis, B. cereus and S. aureus as well as in F. nucleatum consensus-like CcpN operators are located upstream of the thyB genes in approximately the same distance as in B. subtilis. In other bacteria, more mismatches were found in the operator sequence and operator spacing compared to B. subtilis. However, it is conceivable that other organisms have other requirements for CcpN consensus operator sites, and that these sites bind their corresponding CcpN proteins quite well.

Overall, it appears that CcpN is involved in the regulation of one more B. subtilis gene than previously anticipated and upcoming searches might reveal more new targets. At least the high degree of conservation regarding binding sites, even between different organisms, strongly favours this hypothesis.

This report demonstrates that bioinformatics in concert with molecular biological methods can be used to identify new targets of a transcriptional regulator, even if the biological context in which this regulator acts on these targets is not yet understood. In the case of thyB, analyses in other firmicutes that have both a CcpN homologue and a CcpN binding site upstream of their thyB genes and encode – in contrast to B. subtilis – only one thymidylate synthetase, might shed light on a possible role of CcpN-mediated regulation of these genes.
Acknowledgements

We thank M. Fujita for the strains for expression of His-tagged B. subtilis RNA polymerase and sigma factor. Furthermore, we thank C. Wiedemann for helping with the measurements of the lacZ fusions. This work was supported by grant BR1552/6-2 from Deutsche Forschungsgemeinschaft (to S. B.). A. L. is financed by a scholarship from the federal state of Thuringia and from Deutsche Forschungsgemeinschaft.

References


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**Figure Legends**

**Figure 1**  
*Determination of the intracellular concentration of CcpN*  
Western Blot of a 12 % SDS polyacrylamide gel. 1-3: parallels of protein crude extract of *B. subtilis* DB104, corresponding to 1.2 × 10^8 cells; 4: 2.2 pmol of purified CcpN-His_5; 5: 4.4 pmol of purified CcpN-His_5; 6: 1.4 pmol of purified CcpN-His_5 in protein crude extract of DB104 (*ccpN::cat*); 7: 5 pmol BSA. The blot was developed using horseradish peroxidase catalysed conversion of diaminobenzidine, PC-BAS 2.08e software was used for quantification. M: band corresponding to the protein monomer; D: band corresponding to a putative protein dimer.

**Figure 2**  
*EMSA with different putative CcpN targets*  
EMSAs of double-stranded 23 bp DNA fragments containing the consensus binding site of the *sr1*, *geaD*, *thyB* and *yhaM* operators. The DNA was incubated with increasing concentrations of purified CcpN-His_5 (CcpN concentration from left to right: 0 nM; 99 nM; 296 nM; 889 nM, 2.67 µM and 8 µM). The autoradiograms of the gels are shown.

**Figure 3**  
*DNase I footprinting and interference footprinting of the thyB promoter*  
(a) DNase I footprint. GA + CT: sequencing reaction; -: control without DNase I. The DNA was incubated with increasing concentrations of purified CcpN-His_5 (CcpN concentration from left to right: 0 nM; 296 nM; 889 nM, 2.67 µM and 8 µM) prior to DNase I treatment. The combined protected sites have been designated site I+II. The autoradiograms of the gels are shown. Left, wild-type *thyB* promoter; right, mutated *thyB* promoter. An Overview of the protected region at the *thyB* promoter is shown below the gels. Bases protected by CcpN are coloured with a grey background. ~35 and ~10 regions, the transcriptional start site, the CcpN-operator consensus and the mutated operator sequence used in *lacZ* fusions (mutation shown in inverted colours) are indicated.  
(b) Methylation interference footprint of the *thyB* promoter. CT: Maxam-Gilbert C+T sequencing reaction; C, control (protein-free methylated DNA); B and U, bound and unbound fraction of methylated DNA subjected to binding with CcpN-His_5. Close contacts are indicated by black triangles.  
(c) KMnO_4 interference footprint of the *thyB* promoter. Abbreviations are as in C. GA: Maxam-Gilbert G>A sequencing reaction; C, control (protein-free KMnO_4-treated DNA).
(d) Column diagrams indicating the relative strength of interference signals for both strands of the three operators. Only positive signals, i.e. signals that indicate contacts, are shown. Measured values are averaged from four independent experiments.

Figure 4

A. $\beta$-galactosidase assays

Column diagrams showing the amount of Miller units measured at the thyB (with wild type or mutated CcpN operator) promoter under repressing and non-repressing conditions in the DB104 wild-type and ccpN knockout strains. The cells were grown in SP medium till an OD$_{600}$ of 2.0. Values are averaged over 6 different clones and three independent experiments.

B. In vitro transcription at the thyB promoter

In vitro transcription assay using 100 nM of a DNA fragment and 50 nM of His-tagged B. subtilis RNAP as well as 50 nM purified SigA in each reaction. The autoradiogram of the gel is shown. Reaction conditions are denoted above each lane and the thyB transcript is indicated by a black arrow. One of three independent experiments is shown. LC, loading control.

Figure 5

Alignment of promoter regions

Alignment showing the regions upstream of the translational start site of thyB of different bacteria possessing a ccpN homologue or orthologue. The translational start site is indicated in bold and underlined. Sequences resembling the B. subtilis consensus binding site for CcpN have been labelled in bold and with black rectangles. Deviations from the consensus sequence are marked by grey letters.

Table 1: Strains and plasmids used in this study

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<td>F- $\varphi$80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK- mK') phoA supE44 $\lambda$-thi-1 gyrA96 relA1</td>
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Figure 1 Eckart et al.
Figure 2 Eckart et al.
Manuskript IV

Site I+II

CcpN-His

GACTATAAAAAAATCATTTCTGGGTTCAGAAATGATTTTTTAT ACTA

CGACTGATATTTTTTTAGTAAAGACCCAAGTCTTTACTAAAAAATAACACAATGTGATGAT

TGTGTTACACT -35 -10

TGTGHYATACT

CcpN-operator consensus

TGT TTACACT

mutated operator used for fusions

lacZ

Figure 3 Eckart et al.
(a) **thyB**

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<tr>
<td></td>
<td>wild type operator</td>
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![Graph showing Miller units for different glucose concentrations and conditions](image)

(b) **thyB RNA Amount**

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<th>+</th>
<th>-</th>
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Figure 4 Eckart et al.
Manuskript IV

B. subtilis

ATG

ATACAGAA GACTACTTTT AAAGGATGAA AAAA

B. amyloliquefaciens

ATG

ATGTTAAC ACTACTAAA GACTACTTTA AAAGGATGAA AAAA

B. anthracis

ATG

ATGATATA ACTCATATA AGATTGAAG AAGGTTTTAC TACATA

B. cereus

ATG

ATGATATA ACTCATATA GACTAGAGAT TTGAAAGAG GCCCTTCTAC ATG

B. halodurans

ATG

ATGCTAATG AAGGATCATA AGCAGATGG ATG

F. nucleatum

ATG

ATGTTAAC ACTACTAAA GACTACTTTA AAAGGATGAA AAAA

O. iheyensis

ATG

ATGATATA ACTCATATA AGATTGAAG AAGGTTTTAC TACATA

S. aureus

ATG

ATGATATA ACTACTAAA GACTACTTTA AAAGGATGAA AAAA

S. epidermitis

ATG

ATGATATA ACTACTAAA GACTACTTTA AAAGGATGAA AAAA

S. agalactiae

ATG

ATGATATA ACTACTAAA GACTACTTTA AAAGGATGAA AAAA

Figure 5 Eckart et al.
7. Diskussion


7.1. CcpN-DNA-Interaktionsanalyse

7.1.1. Bindungsmuster und Operatorverteilung von CcpN

In dieser Arbeit wurde eine hochauflösende Bestimmung der für eine CcpN-Bindung nötigen Basen mittels Interferenz-Footprinting an den einzelnen Operatoren durchgeführt. Dabei stellte sich heraus, dass CcpN an allen drei untersuchten Promotoren zwei Bindungsstellen besetzt, von denen jeweils eine stärker als die andere kontaktiert wurde. Es konnte auch für den *pckA*-Operator, der im DNase-I-Footprint nur eine ausgedehnte Bindungsstelle aufwies (Servant *et al.*, 2005), gezeigt werden, dass zwei deutlich voneinander getrennte Operatoren existieren. Allen Operatoren an den drei Promotoren ist gemein, dass die am intensivsten kontaktierten Basen Guanin und Thymin sind. Zu Adenin-Resten wurden mit einigen Ausnahmen in der Mehrzahl nur schwächere Kontakte detektiert, zu Cytosin-


7.1.2. Kooperative Bindung von CcpN


Abbildung 5: Übersicht über CcpN-DNA-Kontakte

Interessanterweise liegen die Operatoren für CcpN an den drei Promotoren immer ein ganzzahliges Vielfaches einer Helixwindung auseinander, zwei am *pckA*-Promotor und drei am *sr1*- und *gapB*-Promotor. Da die kooperative Bindung von CcpN eine Interaktion der an den einzelnen Operatoren gebundenen Proteine nahelegt, ist es wahrscheinlich, dass am *sr1*- und am *gapB*-Promotor eine Biegung der DNA stattfindet, um die beiden Repressoren in räumliche Nähe zueinander zu bringen.

### 7.1.3. Energetische Betrachtung der CcpN-DNA-Interaktion


### 7.2. Identifizierung der Regulatoren von CcpN

#### 7.2.1. Aktivierung der CcpN-Aktivität

Bereits eine erste Charakterisierung des *ccpN*-Gens zeigte, dass dessen Expression nicht reguliert ist (Servant *et al.*, 2005), was zu einer konstanten CcpN-Konzentration in der Zelle führt. Da die CcpN-vermittelte Repression allerdings nur unter glycolytischen Bedingungen erfolgen darf, ist ein Regulator für CcpN erforderlich, der dessen Aktivität moduliert. In dieser Arbeit wird die Suche und Identifizierung intrazellulärer Modulatoren der CcpN-Aktivität beschrieben. Mit Hilfe von *in vitro*-Transkriptions-Assays wurde gezeigt, dass CcpN
ohne zusätzliche Faktoren in sehr hohen Konzentrationen spezifisch die Transkription an den kontrollierten Promotoren reprimieren kann, während Kontroll-Promotoren nicht betroffen waren. EMSAs, die mit Protein-Rohextrakten aus *B. subtilis* durchgeführt wurden und in denen signifikante Mengen an Protein-DNA-Komplex detektiert werden konnten (Licht et al., 2005) liefern einen Hinweis darauf, dass die CcpN-Konzentration in der Zelle relativ hoch sein muss. Dies und die beobachtete Modulator-unabhängige Repression erlauben es, die starke Derepression des pckA- und gapB-Gens zu erklären, die von Servant et al. in einem *ccpN*-Knockout-Stamm beobachtet wurden und liefern zudem einen Hinweis darauf, dass CcpN auch in vivo ständig an seine Operatoren gebunden ist. Transkriptionsfaktoren, die ihre Operatoren konstitutiv besetzen, sind nicht ungewöhnlich. Ähnliche Beobachtungen wurden auch für das ResD-Protein aus *B. subtilis* (Härtig et al., 2004), das das yclJK-Operon bei Sauerstoff-Mangel induziert oder das NorR-Protein aus *E. coli* berichtet (Tucker et al., 2006). Allerdings üben diese beiden Proteine, im Gegensatz zu CcpN, keine konstitutive Repression an den von ihnen regulierten Promotoren aus. Eine geringe Derepression (1,3-fach) in einem Repressor-Knockout-Stamm wird häufig berichtet, wie z. B. beim BzdR-Repressor aus *Azoarcus* sp. CIB (Barragá et al., 2005), allerdings ist eine derart starke konstitutive Repression, wie sie bei CcpN beobachtet wurde (5,4-fach) sehr ungewöhnlich und ihre physiologische Relevanz beim derzeitigen Erkenntnisstand nicht erklärbar.


Durch eine Domänenanalyse der CcpN-Sequenz wurde festgestellt, dass CcpN zwei CBS-Domänen besitzt. Für diese nach der Cystathion-β-Synthetase benannten Domänen konnte die Bindung des Adenin-Restes verschiedener Nucleotide und Nucleoside nachgewiesen werden (Scott et al., 2004). Eine Untersuchung des Einflusses von ATP auf die Repressionseffizienz von CcpN in *in vitro* zeigte zwar eine generelle und unspezifische Verbesserung der Transkriptionseffizienz, jedoch nur einen äußerst geringen spezifischen Effekt auf die

7.2.2. Repression der CcpN-Aktivität


7.2.3. Untersuchung einer CcpN-Mutante

Um diese Befunde zu untermauern, wurde ein CcpN-Protein mit einer Mutation in einem konservierten Rest innerhalb einer der CBS-Domänen untersucht. Aymerich und Declerck haben beobachtet, dass dieses Protein in vivo nicht mehr aktiv ist, aber dieselbe Struktur wie natives CcpN besitzt (Chaix et al., Manuskript in Vorbereitung). Die hier durchgeführten in vitro-Transkriptionen, in denen das Protein wie der Wildtyp eine effektorunabhängige Repression vermitteln kann, bestätigen diesen Befund und zeigen außerdem, dass die DNA-
Bindungsfähigkeit durch die Mutation nicht beeinträchtigt wurde. Interessanterweise reagiert diese Mutante nicht mehr auf ATP in in vitro-Transkriptions-Versuchen, und Aymerich und Declerck konnten außerdem zeigen, dass diese Mutante kein ATP oder ADP mehr binden kann. Damit wurde zum einen ein für die ATP-Bindung wichtiger Rest in CcpN identifiziert und außerdem gezeigt, dass eine direkte ATP-Bindung durch CcpN für eine effiziente Repression essentiell ist.

Zusammenfassend lässt sich feststellen, dass ATP und niedriger pH-Wert die intrazellulären Signale sind, die die Repressionseffizienz von CcpN steuern. Durch EMSAs konnte in dieser Arbeit gezeigt werden, dass diese Effektoren die Affinität von CcpN zur DNA allerdings nicht verändern, was darauf schließen lässt, dass CcpN seine Operatoren in vivo ständig besetzt und nur durch eine induzierte Strukturänderung als Metabolismusspezifischer Repressor aktiv werden kann. Diese Eigenschaft findet man auch bei anderen Transkriptionsfaktoren, so z. B. bei NorR aus E. coli (Tucker et al., 2006) oder ResD aus B. subtilis, welches ständig an seinen Operator gebunden ist und erst durch eine Phosphorylierung als Repressor aktiv wird (Härtig et al., 2004).

7.3. Aufklärung des Repressionsmechanismus von CcpN

7.3.1. Repressionsmechanismus von CcpN


Als interessant hervorzuheben ist allerdings, dass eine gewisse Konkurrenz zwischen CcpN und der RNAP bezüglich der Promotorbindung besteht. Es wurde gezeigt, dass die Intensitäten aller Protein-DNA-Komplexe im EMSA deutlich abnahmen, sobald sowohl CcpN als auch die RNAP im Ansatz vorhanden waren. Da die Konzentrationen von CcpN und der RNAP so gewählt wurden, dass sie den Konzentrationen in vivo entsprechen (Wagner, 2000; Manuskript IV) kann davon ausgegangen werden, dass auch in vivo eine Konkurrenz bei der Bindung CcpN-regulierter Promotoren besteht. Dies erklärt auch die
Diskussion


Repressoren, die gleichzeitig mit der RNAP an einen Promotor binden können, reprimieren oft die Bildung des offenen Komplexes, also das Entwinden der DNA-Helix. Beispiele dafür sind der Repressor MerR aus *E. coli* am merT-Promotor (Heltzel *et al.*, 1990; Summers, 1992) oder das Protein KorB des Plasmids RK2 (Williams *et al.*, 1993). Während der Operator von MerR mit dem der RNAP überlappt, ist dies bei KorB nicht der Fall. Beide Situationen finden sich auch an den CcpN-regulierten Promotoren, allerdings konnte durch die „Open-complex-formation-Assays“ ausgeschlossen werden, dass die Bildung des offenen Komplexes an den drei Promotoren beeinträchtigt ist.


Am pckA- und sr1-Promotor hingegen konnte die Synthese abortiver Transkripte auch unter reprimierenden Bedingungen detektiert werden, allerdings wird der Übergang in einen produktiven Elongationskomplex, also das Verlassen des Promotors durch die RNAP, an diesen Promotoren inhibiert. Dies kann durch zwei mögliche Mechanismen geschehen. Die erste Möglichkeit wäre ein Repressor, der downstream der RNAP bindet und so eine physische Blockade darstellt. Die H-NS-vermittelte Repression des eltAB-Operons in *E. coli* funktioniert beispielsweise nach diesem Prinzip (Yang *et al.*, 2005). Betrachtet man allerdings die Position der Operatoren am pckA- und sr1-Promotor, erscheint diese Art der Repression sehr unwahrscheinlich, was wiederum für die zweite Möglichkeit spricht: Die direkte Interaktion zwischen dem Repressor und der RNA-Polymerase. Es ist bekannt, dass Promotorsequenzen, die in allen Elementen nahezu dem Konsensus entsprechen, den Übergang in einen Elongationskomplex durch eine zu feste Bindung der RNAP verhindern können. Repressoren, die ihre Operatoren in der Regel mit hoher Affinität binden, können
Diskussion

Durch eine Interaktion mit der RNAP diesen Effekt nachahmen und die RNAP so am Promotor arretieren. Beispiele dafür sind das Protein p4 des Phagen Φ29 am A2c-Promotor oder auch der Gal-Repressor (Choy et al., 1995; Monsalve et al., 1996).

7.3.2. CcpN-RNAP-Interaktion


Am gapB-Promotor kann eine Interaktion mit der α-CTD aufgrund der Position der Operatoren und mit dem σ-Faktor aufgrund der Interaktionsexperimente ausgeschlossen werden, jedoch wäre eine Interaktion mit anderen Bereichen der RNAP möglich. Allerdings gibt es nur wenige Proteine, die mit der β- oder β'-Untereinheit interagieren. Ob CcpN tatsächlich mit anderen Teilen der RNAP wechselwirken kann, muss noch experimentell bestätigt oder widerlegt werden, aber die geringe Größe von CcpN, die wenig Platz für mehrere Interaktionsoberflächen bietet und die Tatsache, dass erhebliche Änderungen der DNA-Struktur durch die Bindung von CcpN auftreten, sprechen für die oben genannte Repression durch DNA-Strukturänderungen, wie sie z. B. für H-NS gezeigt werden konnte (Schneider et al., 1999).
Abbildung 6:
Aktuelles Modell der CcpN-Aktivität unter glycolytischen Bedingungen


(b): Situation am pckA-Promotor. Die Situation stellt sich hier, abgesehen von leicht veränderten Operator-Positionen, ähnlich der am sr1-Promotor dar.


### 7.4. Suche nach neuen CcpN-Zielgenen

#### 7.4.1. Die intrazellulare Konzentration von CcpN und Suche nach neuen Zielgenen

ihnen regulierten Gene und Operons zu korrelieren: Während der LacI-Repressor nur ein
Operon kontrolliert, regulieren CcpA und CodY jeweils ca. 100 Gene oder Operons
(Sonenshein, 2005; Sonenshein, 2007). Diese Beobachtung war Ausgangspunkt, um neue
Zielgene von CcpN zu suchen und zu identifizieren.

Dazu wurde im Genom von *B. subtilis* nach neuen CcpN-Operatoren gesucht und 22
mögliche Operatoren in Promotornähe entdeckt, die der Konsensussequenz entsprachen sowie
291 mögliche Operatoren, die eine Abweichung vom Konsensus aufwiesen. Von diesen
Operatoren zeigten nur drei eine spezifische Bindung von CcpN: Diese lagen upstream der
Gene *thyB*, *yhaM* und *gcaD*. Alle diese Operatoren banden CcpN teilweise deutlich schlechter
als der starke *sr1*-Operator.

ThyB codiert eine Neben-Thymidylat-Synthase in *B. subtilis*, die für 5 % der Thymidylat-
Synthese in diesem Organismus verantwortlich ist, die restlichen 95 % werden von ThyA
synthetisiert (Neuhard *et al.*, 1978). *B. subtilis* stellt in dieser Hinsicht eine Besonderheit dar,
da es der einzige Organismus ist, in dem zwei Thymidylat-Synthasen gefunden wurden. Die
hier untersuchte ThyB ist ein Homologes der in anderen Bakterien vorkommenden
Thymidylat-Synthase, während die aktivere ThyA der Thymidylat-Synthase aus Phagen
ähnelt (Tam & Borriss, 1998). YhaM wurde als 5’-3’-Exoribonuclease identifiziert, die durch
Magnesium- und Cobalt-Ionen aktiviert und durch Mangan-Ionen inhibiert wird (Oussenko
*et al.*, 2002). Sie spielt vermutlich eine Rolle während der DNA-Replikation (Noirot-Gros
*et al.*, 2002). GcaD codiert die N-Acetyl-Glucosamin-Pyrophosphorylase, ein Enzym, das an der
Biosynthese von Peptidoglycan und Lipopolysacchariden und damit letztendlich am
Zellwandaufbau beteiligt ist (Hove-Jensen, 1992). Da die Interaktion von CcpN mit den
*yhaM*- und *gcaD*-Operatoren äußerst schwach war, wurde nur der *thyB*-Operator weiter
untersucht.

7.4.2. CcpN-vermittelte Regulation von *thyB*

DNase I-Footprints zeigten, ähnlich wie am *pckA*-Promotor, nur eine zusammenhängende
geschützte Region, die sowohl die –10- als auch die –35-Box umfasst. Interessanterweise
war der Schutz vor DNase I-Abbau über den gesamten Footprint nicht konstant wie am *sr1*-,
*gapB*- und *pckA*-Promotor, sondern innerhalb der Konsensus-Sequenz deutlich intensiver.
Auch Interferenz-Footprints zeigten eine neuartige Kontaktverteilung am *thyB*-Promotor mit
den stärksten Kontakten innerhalb des Konsensus-Operators und schwächeren Kontakten in
zwei zusätzlichen Operatoren, während an allen anderen CcpN-regulierten Promotoren nur
eine starke und eine schwache Bindungsstelle vorhanden ist. Zum am weitesten upstream gelegenen Operator am thyB-Promotor werden fast ausschließlich Kontakte zu Adenin-Resten ausgebildet. Da diese Interferenz-Footprinting-Methode Kontakte zu Adeninen in der kleinen Furche detektiert, kann man daraus schließenfolgern, dass CcpN hier einen DNA-Kontakt über die kleine Furche herstellt. Kontakte in der kleinen Furche finden oft in Form von indirektem Auslesen, d. h. Erkennung von DNA-Strukturen anstatt Sequenzen, statt, wie z. B. bei CRP oder HU in E. coli (Swinger & Rice, 2007; Lindemose et al., 2008), oder dienen oft nur der Stabilisierung von Protein-DNA-Interaktionen, da die kleine Furche nur einen geringen Informationsgehalt aufweist.


8. Zusammenfassung/Summary

8.1. Zusammenfassung

Im Rahmen dieser Arbeit wurde der Transkriptionsfaktor CcpN aus dem Gram-positiven Bakterium *Bacillus subtilis*, der die Gene *sr1*, *pckA* und *gapB* reguliert, eingehend charakterisiert.


Da CcpN in konstanter Menge in der Zelle vorhanden und seine Expression nicht reguliert ist, wurde nach intrazellulären Effektoeren für CcpN gesucht. Es konnte gezeigt werden, dass CcpN auch ohne Effektoeren die drei untersuchten Promotoren spezifisch, jedoch unreguliert, reprimieren kann, was eine Erklärung für die starke Derepression der CcpN-regulierten Gene in einem *ccpN*-Knockout-Stamm liefert. Weiterhin konnten mit Hilfe von *in vitro*-Transkriptions-Versuchen ATP und niedriger pH-Wert als positive Effektoeren von CcpN identifiziert werden. Diese Ergebnisse fügen sich gut in die beobachtete Aktivität von CcpN *in vivo* ein, das nur unter glycolytischen Bedingungen als Repressor wirkt, welche in der Regel mit hohem ATP-Spiegel und leicht gesenktsem pH-Wert aufgrund der Acetat-

Da in der CcpN-Sequenz zwei CBS-Domänen existieren, für die in der Vergangenheit die Bindung von Adenosin-Resten gezeigt werden konnte, wurde eine CcpN-Mutante untersucht, bei der eine konservierte Aminosäure innerhalb einer CBS-Domäne durch ein Alanin ausgetauscht wurde. Diese Mutante, die dieselbe Struktur wie der Wildtyp besaß und auch noch in der Lage war, DNA zu binden, reagierte in der in vitro-Transkription nicht mehr auf ATP, wodurch gezeigt wurde, dass die CBS-Domänen auch bei CcpN für die ATP-Bindung verantwortlich sind.

Letztendlich wurde noch mit Hilfe von EMSAs demonstriert, dass ATP und niedriger pH-Wert die Affinität von CcpN zu seinen Operatoren nicht ändern können.


Interaktionssstudien mittels Far-Western-Blot und Co-Elutions-Versuchen zeigten, dass CcpN in der Lage ist, mit der α-Untereinheit der RNAP, nicht jedoch mit dem σ-Faktor, zu interagieren. Dies legte nahe, dass die Inhibition des Übergangs in den Elongationskomplex am sr1- und pckA-Promotor durch Arretierung der RNAP am Promotor, vermittelt durch eine
Interaktion zwischen CcpN und der α-Untereinheit, erfolgt. Am gapB-Promotor besteht diese Möglichkeit aufgrund der Position der Operatoren nicht, doch wurde an diesem Promotor in vorangegangenen Arbeiten eine starke Änderung der DNA-Struktur bei der Bindung von CcpN gezeigt, durch die die Transkriptionsinitiation blockiert werden könnte.


8.2. Summary

The scope of this work was the detailed characterisation of the transcription factor CcpN of the Gram-positive bacterium *B. subtilis* which regulates the genes *sr1*, *pckA* and *gapB*.

To this end, bases within the operators that are necessary for forming contacts with CcpN were determined at the *sr1*, *pckA* and *gapB* promoters by interference footprinting. These experiments showed that intensive contacts were made within a sequence corresponding to the previously determined consensus sequence and that each promoter consists of two operator segments with different contact strength. EMSAs demonstrated that these differences in contact strength also resulted in a varying binding strength of the single operators when present on a separate DNA fragments. However, if both operators were located on one DNA fragment, both were bound with equal strength and in addition more intensively than the single operator sites. Regarding the change in shape and slope of the binding curves when comparing single operators with operator pairs and the energy gain resulting from CcpN-operator interaction it was concluded that CcpN binds its operator sites cooperatively. Furthermore, energetic calculations of CcpN-DNA interaction at different temperatures revealed that the binding process is driven by a strong enthalpy rather than strong entropy, ensuring a stable interaction of CcpN with its operators over a large temperature scale.

Since CcpN is present in constant concentrations within the cell and the expression of its gene was found to be not regulated, a search for intracellular effectors of CcpN was performed. *In vitro* transcription reactions showed that purified CcpN without an effector is able to specifically repress transcription at the three investigated promoters and provides an explanation for the strong derepression observed in a *ccpN* knockout strain. ATP and low pH were identified as the intracellular activators of CcpN activity, fitting quite well into CcpN’s scheme of action: During glycolysis, when CcpN is active, ATP levels in the cell are high and the cytosol becomes slightly acidic due to acetate production. On the contrary, ADP has been shown to counteract the activating effect of ATP at equimolar concentrations. Both results have been substantiated by CD spectroscopy, which showed extensive structural rearrangements of CcpN upon ATP binding at low pH, but not at neutral pH, while ADP binding did only result in weak structural alterations. It was thus concluded that the repression activity of CcpN is stimulated by structural alterations induced by ATP binding at low pH and repressed by ADP binding.
Two CBS domains, which are able to bind adenosine residues, have been found within the CcpN sequence. To elucidate the role of these domains, a CcpN mutant with an amino acid exchange in a conserved residue in one domain was investigated. This mutant, although still being able to bind to DNA and showing the same structure as the wild type, did no longer respond to ATP, indicating that the CBS domains are indeed responsible for ATP binding.

Eventually, EMSAs performed under repressing and non-repressing conditions showed that the positive effectors of CcpN did not alter the affinity to its operators.

Since nothing was known about the repression mechanism of CcpN, efforts have been made to elucidate this mechanism at the three investigated promoters. EMSAs have demonstrated that CcpN and RNAP are able to bind together to the promoter under repressive and non-repressive conditions. However, competition for promoter binding between these two proteins has been observed which explains the strong derepression in a ccpN knockout strain. Using open complex formation assays it has been demonstrated that open complexes can still be formed under repressive conditions at all three promoters, showing that CcpN does not prevent melting of the DNA. At the gapB promoter, no abortive transcripts were detectable under repressive conditions indicating that CcpN represses transcription initiation at this promoter. The sr1 and pckA promoters still showed abortive transcript synthesis under repressive conditions, but the transition to the elongation complex was inhibited.

Far western blot and co-elution interaction studies showed that CcpN is able to specifically interact with the α-subunit of RNAP, but not with the σ-factor. This suggests that the repression at the sr1 and pckA promoters occurs by interaction between CcpN and the α-subunit, which in turn stalls the RNAP at the promoter. This mechanism can, however, be excluded for the gapB promoter, since the operator location does not allow CcpN to be positioned in a way to properly contact the α-subunit. Here, an alteration in DNA structure upon CcpN binding has been detected in preceding investigations, which is potentially responsible for the prevention of transcription initiation.

Early characterisations of CcpN already suggested a rather high intracellular concentration. This observation was substantiated using western blots, and the amount of CcpN was determined to be 4000 molecules per cell. Since there is a certain correlation between repressor concentration and the number of regulated genes, a search for new CcpN target genes was performed in the genome of B. subtilis. The search resulted in numerous potential operators, but only those located in the promoter region of the thyB, yhaM and gcaD genes.
showed CcpN binding \textit{in vivo}, with \textit{thyB} being the only one strong enough to be investigated further. Interestingly, the \textit{thyB} CcpN operator showed a binding pattern not observed before, where one strong and two weak operators are conjoined without a spacer region.

Using \textit{lacZ} transcriptional fusions and \textit{in vitro} transcription, a slight activation of the \textit{thyB} promoter by CcpN under glycolytic conditions has been shown. This regulation seems to be of little physiological relevance in \textit{B. subtilis}, since the \textit{thyB} gene is hardly active, and thymidylate synthase activity is mainly carried out by ThyA. However, related species containing only a \textit{thyB} and no \textit{thyA} gene have CcpN operator sequences upstream of their respective \textit{thyB} genes and it is feasible that CcpN plays an important regulatory role in \textit{thyB} expression in these bacteria.

The results of the presented study greatly increase our understanding of the transcription factor CcpN from \textit{B. subtilis}. This makes – together with preceding works – CcpN to one of the best characterised transcription factors in \textit{B. subtilis} including one of the few for which a repression mechanism has been determined.
9. Literaturverzeichnis


Danksagung

Als erstes möchte ich mich bei HD Dr. Sabine Brantl für die Überlassung des interessanten Themas und die Möglichkeit, die Arbeit in den Labors der Arbeitsgruppe Bakteriengenetik durchführen zu können, bedanken. Weiterhin gebührt ihr mein Dank für die ständige Diskussionsbereitschaft, die wertvollen konstruktiven Hinweise und Ratschläge und natürlich die hervorragende Betreuung während meiner Arbeit.

Prof. Dr. Frank Große und Prof. Dr. Wolfgang Hillen möchte ich für die Anfertigung der Gutachten für die vorliegende Dissertation danken.

Mein Dank gilt außerdem Dr. Ralph Golbik von der Martin-Luther-Universität Halle-Wittenberg, der mir die Möglichkeit gab, am dortigen CD-Spektrometer Messungen durchzuführen und dessen fachliche Kenntnisse einen wichtigen Teil zur Identifikation der CcpN-Regulatoren beitrugen.

Stéphane Aymerich und Nathalie Declerck möchte ich für anregende Diskussionen zum Thema CcpN sowie für die Zurverfügungstellung von Forschungsdaten und Plasmiden danken.

Den gegenwärtigen und ehemaligen Mitgliedern der Arbeitsgruppe Bakteriengenetik, namentlich Heike Preis, Nadja Heidrich, Christina Stacke, Rita Eckart, Matthias Gimpel, Christoph Wiedemann und Alberto Chinali, gebührt mein Dank für die angenehme Laboratmosphäre und viele produktive Gespräche und Ideen.

Abschließend möchte ich noch meiner Familie, insbesondere meinen Eltern, und im ganz Speziellen meiner Freundin Verena Wolf für die während meiner Arbeit entgegengebrachte Unterstützung ganz herzlich danken.
Selbstständigkeitserklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Jena, 14.08.2009

Erklärung zur Bewerbung

Ich erkläre, dass ich mich mit der vorliegenden Arbeit an keiner anderen Hochschule um den akademischen Grad Dr. rer. nat. beworben habe und dass ich weder früher noch gegenwärtig die Eröffnung eines Verfahrens zum Erwerb des o. g. akademischen Grades an einer anderen Hochschule beantragt habe.

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Thesen zur Dissertation

1. Im Rahmen dieser Arbeit wurde der Transkriptionsfaktor CcpN aus dem Bakterium *Bacillus subtilis* eingehend charakterisiert.
2. CcpN besetzt an den Promotoren der Gene *sr1*, *pckA* und *gapB* jeweils zwei Operatoren, die zwei (am *pckA*-Promotor) oder drei (am *sr1*- und *gapB*-Promotor) Helixwindungen auseinander liegen.
3. Einer der Operatoren wird dabei jeweils stärker kontaktiert und – wenn isoliert vorhanden – auch stärker gebunden als der andere, wobei die Sequenz dieses Operators in allen Fällen deutlich näher an der Konsensussequenz als die des anderen Operators ist.
4. Sind beide Operatoren auf einem DNA-Fragment vorhanden, werden sie von CcpN gleich stark gebunden, die dabei im Vergleich zur Bindung der Einzeloperatoren freiwerdende Energie und die Änderung der Bindungskurven zeigen, dass CcpN die DNA kooperativ bindet.
5. Die Bestimmung der freien Bindungsenergie bei unterschiedlichen Temperaturen zeigt, dass die CcpN-DNA-Interaktion enthalpisch getrieben wird.
6. Da die Expression von CcpN nicht reguliert wird, muss es einen intrazellulären Regulator geben, der die Aktivität in Abhängigkeit von der Stoffwechselsituation modifiziert.
7. CcpN kann in hoher Konzentration auch ohne Regulator die Expression am *sr1-, pckA- und gapB*-Promotor spezifisch reprimieren.
8. ATP und niedriger pH-Wert sind die intrazellulären Effektores von CcpN und erhöhen dessen Repressionsaktivität erheblich, ADP kann dem Effekt von ATP entgegenwirken und senkt die Repressionsaktivität von CcpN.
9. Bei der Bindung von ATP im Sauren ändert sich die Struktur von CcpN erheblich, jedoch nicht bei der Bindung im Neutralen oder bei der Bindung von ADP.
10. CcpN mit einer Mutation in einer CBS-Domäne weist dieselbe Struktur auf wie der Wildtyp und ist noch in der Lage, DNA zu binden, reagiert allerdings nicht mehr auf ATP. Dies zeigt, dass die CBS-Domäne für die ATP-Bindung zuständig ist.
12. CcpN kann auch unter reprimierenden Bedingungen gemeinsam mit der RNA-Polymerase an den *sr1-, pckA- und gapB*-Promotor binden, konkurriert aber sowohl
unter reprimierenden als auch unter nicht-reprimierenden Bedingungen in gleicher Weise mit der RNAP um die Bindungsstellen des Promotors, was die starke Derepression in einem ccpN-Knockout-Stamm erklärt.


16. CcpN ist in der Lage, spezifisch mit der α-Untereinheit der RNA-Polymerase zu interagieren und nutzt diese Interaktion wahrscheinlich, um die RNAP am sr1- und pckA-Promotor zu arretieren.

17. CcpN kann am gapB-Promotor aufgrund der Lage der Operatoren nicht mit der α-Untereinheit interagieren und blockiert die Transkriptionsinitiation hier vermutlich durch Änderungen in der DNA-Struktur.


20. Der thyB-Promotor zeigt eine neuartige Operatorverteilung, bei der eine Hauptoperator und zwei Hilfsoperatoren ohne Spacer aneinandergrenzen.

21. CcpN kann als schwacher Aktivator am thyB-Promotor wirken.

22. In anderen Spezies, die CcpN codieren, finden sich CcpN-Operatoren upstream des entsprechenden thyB-Gens, was auf eine Regulation durch CcpN auch in diesen Bakterien hindeutet.