Von obligater Anaerobiose zur Aerotoleranz – Die oxidative Stressantwort von *Clostridium acetobutylicum*

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1 EINLEITUNG

Als „kleine Tiere, die in Abwesenheit von Sauerstoff leben“ beschrieb Louis PASTEUR 1861 bewegliche Stäbchen, die in anaerober Kultur aktiven Gärungsstoffwechsel betrieben, diesen aber in Gegenwart von Luft einstellten. Diese Beobachtung sowie seine Schlussfolgerung „Fermentation, c’est la vie sans air“, lösten nicht nur das lange bestehende Rätsel fermentativer Prozesse, sondern gelten heute als Geburtsstunde wissenschaftlicher Mikrobiologie (zur Übersicht: WOLFE, 1999; SCHWARTZ, 2001). Es ist sehr wahrscheinlich, dass hier bereits ohne direktes Wissen eine Anreicherung Butyrat-bildender Clostridien beschrieben wurde. So stimmen diese Eigenschaften nahezu exakt mit denen des 1915 von WEIZMANN aus dem Boden isolierten *Clostridium acetobutylicum* überein.

Dieses Gram-positive, obligat anaerobe Eubakterium mit der Fähigkeit zur Bildung von Endosporen ist physiologisch durch einen biphasischen Gärungsstoffwechsel und komplexen Zellzyklus charakterisiert (**Abb. 1**).

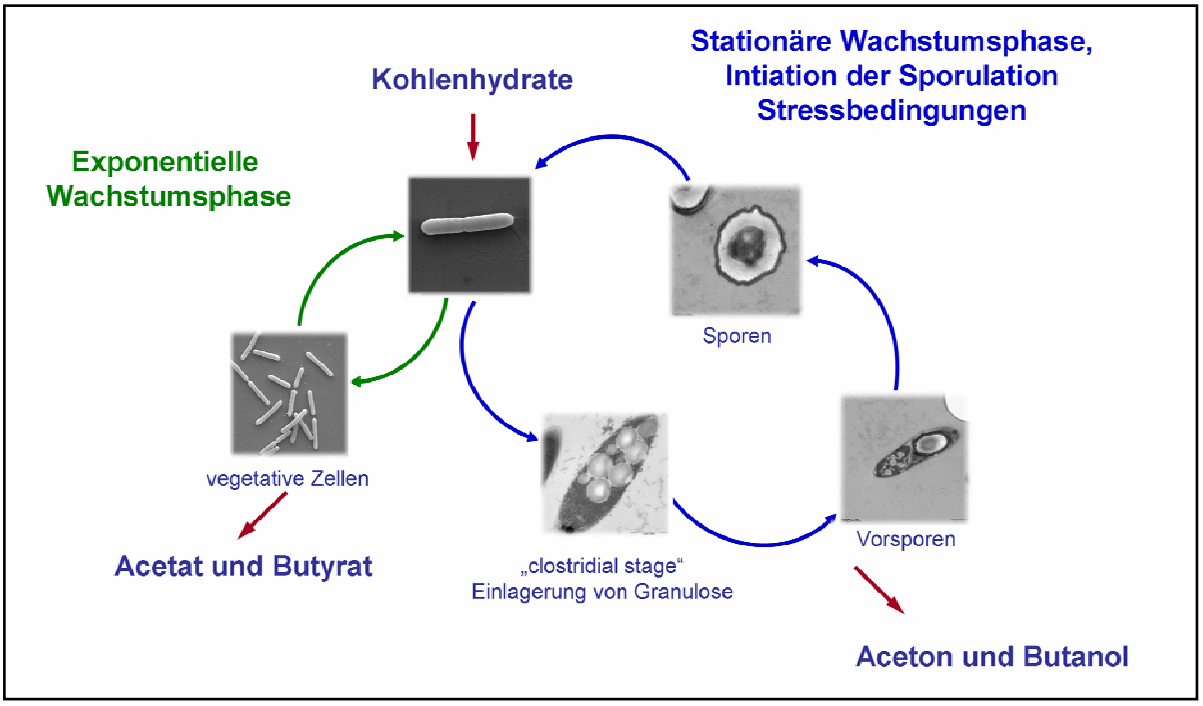

Abb. 1: Zellzyklus mit Produkten der biphasische Gärung von *C. acetobutylicum*

Während seiner exponentiellen Wachstumsphase fermentiert *C. acetobutylicum* Zucker zu den organischen Säuren Butyrat und Acetat. Zusätzlich entstehen Kohlenstoffdioxid und molekularer Wasserstoff. Beim Übergang in die stationäre Phase

kann aber vor der Sporulation eine Umstellung des Stoffwechels stattfinden. Dabei bewirkt u. a. ein Absinken des pH-Wertes in der Umgebung, dass verbliebene Zuckermoleküle, sowie ein Teil der vorhandenen Säuren nun vor allem in die neutralen Lösungsmittel Aceton und Butanol umgewandelt werden. Zeitgleich wird zunächst die Entstehung zigarrenförmiger Zellen („clostridial stage“) und dann die Initiation der Sporulation beobachtet. Das Potential zur biotechnologischen Produktion der beiden letztgenannten Lösungsmittel führte bis zur Mitte des letzten Jahrhunderts zu einer erheblichen wirtschaftliche Bedeutung des Bakteriums (zur Übersicht: BAHL und GOTTSCHALK, 1988; DÜRRE und BAHL, 1996). Heute werden diese Lösungsmittel zwar fast ausschließlich petrochemisch hergestellt, doch ist vor dem Hintergrund steigender Rohölpreise gerade in letzter Zeit die biotechnologische Herstellung von Butanol als Alternativtreibstoff wieder von hohem ökonomischem und wissenschaftlichem Interesse (DÜRRE, 2007 und 2008; DÜRRE und BAHL, 2008). Dieses richtet sich insbesondere auf eine Steigerung der Butanolproduktion. Auch deshalb wurden für *C. acetobutylicum* in der jüngeren Vergangenheit eine Vielzahl von mikro- und molekularbiologischen Methoden etabliert, deren wichtigste in **Tab. 1** zusammengefasst sind.

Tab.1: Mikro- und molekularbiologische Methoden für *C. acetobutylicum*

Methode	Entwickelt bzw. etabliert von
Kultivierung im phosphatlimitierten Chemostaten	BAHL *et al.*, 1982
In vivo Methylierung von Plasmiden zur Elektroporation	MERMELSTEIN und PAPOUTSAKIS, 1993
Sequenzierung des Genoms	NÖLLING *et al.*, 2001
Homo- und heterologe Überexpression von Proteinen	TUMMALA *et al.*, 1999 GIRBAL *et al.*, 2005
Antisense RNA zum „knock down“ gezielter Gene	TUMMALA *et al.*, 2003
Globale Transkriptionsanalyse „microarray“	TOMAS *et al.*, 2003 ALSAKER *et al.*, 2005
Homologe Rekombination zur Deletion gezielter Gene	SOUCAILLE *et al.*, 2006
Gruppe II Introns zur Insertionsmutation gezielter Gene	HEAP *et al.*, 2007

Ihre Anwendung hat bereits maßgeblich zum erweiterten Verständnis der Lösungsmittelbildung und des Zellzyklus von *C. acetobutylicum* beigetragen (JONES *et al.*, 2008; PAPOUTSAKIS, 2008). Aktuelle Arbeiten konzentrieren sich vermehrt auf die Entwicklung von *in silico* Modellen zur funktionellen Gesamtorganisation der Zelle (LEE *et al.*, 2008; SENGER und PAPOUTSAKIS, 2008). Systembiologische Ansätzen, wie das durch das Bundesministerium für Bildung und Forschung geförderte internationale SYSMO (systems biology of microorganisms) Projekt „COSMIC“ (*Clostridium acetobutylicum* systems microbiology), werden daher eine große zukünftige Bedeutung zugesprochen. Unter diesen Gesichtspunkten kann *C. acetobutylicum* nicht nur als Modellorganismus für apathogene Clostridien verstanden werden (YOUNG *et al.*, 1989), sondern eignet sich auch hervorragend für die Untersuchung grundlegender physiologischer Prozesse in anaeroben Organismen.

1.1 Anaerobe Bakterien und Sauerstoff

Wohl kaum ein Faktor hat die frühe Evolution so nachhaltig beeinflusst wie das Auftreten des molekularen Sauerstoffs (O_2), ist er doch eine Grundvoraussetzung für die Entstehung der komplexesten Lebensformen. Erst in den letzen Jahren hat sich manifestiert, dass es nach dem Aufkommen photosynthetisch aktiver Cyanobakterien vor ca. 2,4 Milliarden Jahren zu einem Anstieg in der atmosphärischen Sauerstoffkonzentration kam, die dann, vor ca. 600 Millionen Jahren, nahezu den heutigen Wert erreichte (zur Übersicht siehe KASTING, 2001; KERR, 2005). Die Reaktivität des O_2 mit reduzierten Flavin-Cofaktoren und biologisch wichtigen Übergangsmetallen wie z. B. Eisen führt zur Bildung partiell reduzierter, sehr reaktiver Sauerstoffspezies (ROS) wie Wasserstoffperoxid (H_2O_2), Superoxid (O_2^-) und dem Hydroxyl-Radikal (HO•). Irreversible Schäden an Membranen, Proteinen und der DNA sind die Folge (zur Übersicht: IMLAY, 2003). Dieses Szenario ließ für ein Überleben lediglich zwei Varianten zur Auswahl: entweder eine Anpassung an sich ändernde Bedingungen oder ein Rückzug in O_2-freie Lebensräume. Eine Vielzahl von Organismen perfektionierte diese Anpassung, entwickelte z. B. den Cytochrom *c* Oxidase-Komplex und nutzte die Energie dieses starken Oxidationsmittels (GENNIS und FERGUSON, 1995; TSUKIHARA *et al.*, 1996). Weiterhin entstand eine wirksame enzymatische Abwehr gegen ROS, die weitgehend aus Katalasen und Superoxiddismutasen besteht (**Abb. 2**).

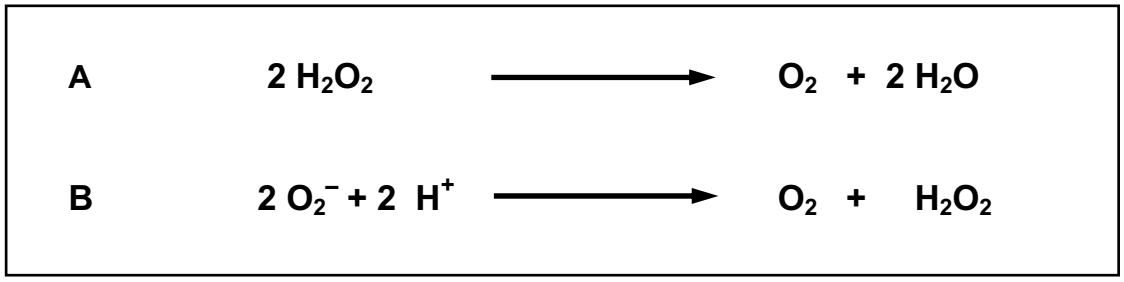

Abb. 2: Disproportionierung reaktiver Sauerstoffspezies durch Katalasen (A) und Superoxiddismutasen (B)

Organismen für die die zweite Variante eingetreten zu sein scheint, werden generell als „anaerob“ klassifiziert. Deren Sauerstofftoleranz kann jedoch stark variieren und diese heterogene Gruppe umfasst sowohl Organismen die schon nach 1 min durch nanomolare O_2-Konzentrationen getötet werden, wie einige methanogene Archaeen, als auch solche, die bis zu 24 h oder länger bei atmosphärischen O_2-Konzentrationen überleben ohne zu wachsen. Letzteres trifft insbesondere auf sulfatreduzierende Eubakterien zu, wie z. B. Vertreter des Genus *Desulfovibrio* (zur Übersicht: BRIOUKHANOV und NETRUSOV, 2007).

1.2 Das traditionelle Modell obligater Anaerobiose und neuere Erkenntnisse

Die Empfindlichkeit obligater Anaerobier gegenüber O_2 wurde lange Zeit der Abwesenheit bzw. Unvollkommenheit einer enzymatischen Abwehr gegenüber ROS zugeschrieben. Tatsächlich schien es bereits in sehr frühen Beobachtungen so, als ob ausschließlich aerobe Organismen in der Lage wären reaktive Sauerstoffspezies zu entgiften (RYWOSCH und RYWOSCH, 1907). Weiterführende Studien bestätigten diese Beobachtungen und führten schließlich zu der Schlussfolgerung, dass eingeschränkte O_2-Toleranz und obligate Anaerobiose eine Konsequenz geringer bzw. fehlender Katalase- und Superoxid-Dismutase (SOD) Aktivitäten wären (McCORD *et al.*, 1971).

Diese Schlussfolgerung ist jedoch gerade während der letzten Jahre immer wieder kritisch hinterfragt worden. So scheint es zum einen unwahrscheinlich, dass lediglich das Fehlen von zwei Enzymen das Leben in anaerobe Nischen zwingt (IMLAY, 2008a),

zum anderen wurden bei einer Vielzahl dieser Vertreter geringe Aktivitäten dieser zwei Enzyme detektiert (zur Übersicht: BRIOUKHANOV *et al.*, 2002). Auch wurden sowohl Katalase- als auch SOD-kodierende Gene in vielen typischen Anaerobiern identifiziert und ihre Proteinprodukte charakterisiert, so z. B. SOD aus *Methanobacterium bryantii* (KIRBY *et al.*, 1981), Katalase aus *Methanosarcina barkeri* (SHIMA *et al.*, 1999) und sowohl SOD als auch Katalase aus *Desulfovibrio gigas* (DOS SANTOS *et al.*, 2000). Gleichzeitig konnte gezeigt werden, dass eine Dismutation zur Entgiftung von O_2^- nicht zwingend notwendig ist, sondern auch effizient durch enzymatische Reduktion durch die neu entdeckten Superoxidreduktasen (SOR) ersetzt werden kann (JENNEY *et al.*, 1999). Diese Entdeckung bildete die Grundlage für erste Modelle zur alternativen Detoxifikation in den obligat anaeroben *Pyrococcus furiosus* und *C. acetobutylicum* (GRUNDEN *et al.*, 2005; RIEBE *et al.*, 2009; **Abb. 3**).

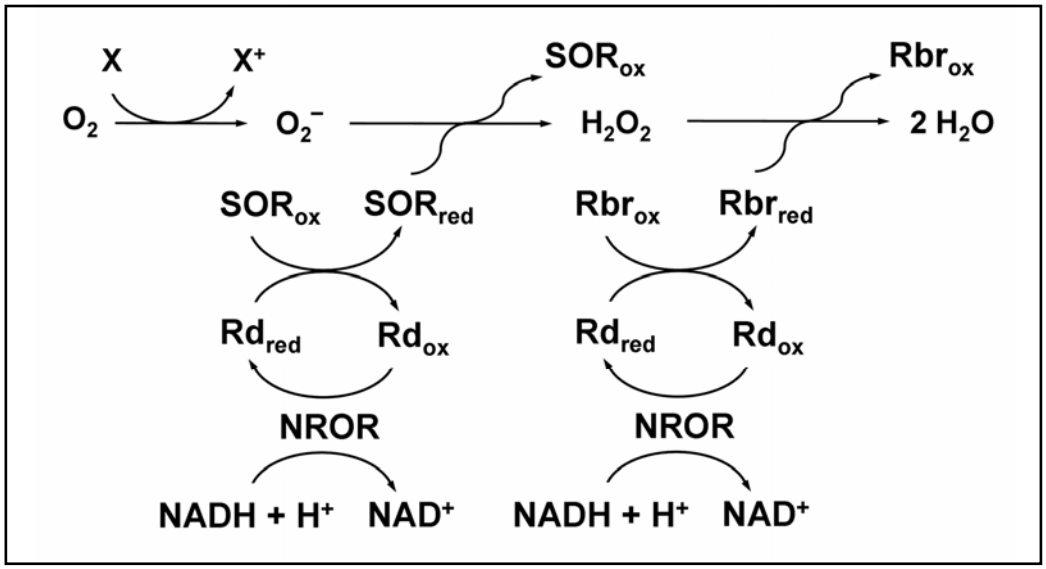

Abb. 3: Reduktive Detoxifizierung von O_2 und reaktiven O_2-Spezies (nach GRUNDEN *et al.*, 2005 und RIEBE *et al.*, 2009; mod.)
NROR, NADH:Rubredoxin Oxidoreduktase; **Rbr**, Rubrerythrin; **Rd**, Rubredoxin; **SOR**, Superoxidreduktase; **X**, unspezifische Elektronendonatoren und Übergangsmetalle; **ox**, oxidiert; **red**, reduziert.

Hierbei werden Elektronen schrittweise von Reduktionsäquivalenten wie NADH oder NADPH über das Redoxcarrierprotein Rubredoxin (Rd) auf partiell reduzierte Sauerstoffspezies übertragen. Reduziertes Rubredoxin wird dabei kontinuierlich durch

eine NAD(P)H-abhängige Rubredoxin-Oxidoreduktase regeneriert. Terminale, Rubredoxin-abhängige Elektronenüberträger sind Superoxidreduktasen, wie Desulfoferrodoxin (O_2^-) oder Peroxidasen, wie Rubrerythrine (H_2O_2). Die finalen Reaktionsprodukte sind H_2O und NAD^+. Ein Vorteil dieser Reaktion ist das hierbei, im Gegensatz zu Dismutationen von H_2O_2 und O_2^-, kein molekularer Sauerstoff entsteht (**Abb. 2** und **Abb. 3**).

Neueren Erkenntnissen zufolge sind es daher weniger ROS, sondern gerade O_2 selbst der von zahlreichen Organismen obligate Anaerobiose verlangt (IMLAY, 2008a). Anaerobier operieren häufig an hoch reduzierten Verbindungen und nutzen dazu Redoxenzyme mit oxidationslabilen Fe-S-Clustern (Pyruvat-Ferredoxin-Oxido-reduktase, Nitrogenase, Kohlenmonoxid-Dehydrogenase und Dehydratasen) oder Glycylradikalen (Pyruvat-Formiat-Lyase). Diese Enzyme ermöglichen zwar Elektronentransport bei sehr niedrigen Redoxpotenzialen, werden aber in Anwesenheit von O_2 irreversibel zerstört (MEINECKE *et al.*, 1989; SAWERS und WATSON, 1998; PAN und IMLAY, 2001). Gerade die Verwendung dieser hoch spezialisierten Enzyme wird seit kurzem als ein limitierender Faktor der O_2-Toleranz in Prokaryoten angesehen (IMLAY, 2003 und 2006).

1.3 *Clostridium acetobutylicum* und obligate Anaerobiose

Eine der am weitesten akzeptierten Eigenschaften der gesamten Gruppe der Clostridien ist, dass alle Mitglieder als obligat anaerob gelten. Doch auch hier variiert der Grad der Sauerstofftoleranz von eher aerotoleranten Genera wie *C. sporogenes, C. perfringens*, und *C. histolyticum* bis zu äußerst empfindlichen wie *C. difficile* und *C. pasteurianum* (MORRIS und O'BRIEN, 1971; BRIOUKHANOV und NETRUSOV, 2007). *C. acetobutylicum* ist in dieser Hinsicht ein gemäßigter Vertreter, der niedrige O_2-Konzentrationen (40-50 µM) für eine gewisse Zeit (4-6 h) überlebt, jedoch ohne dabei aktiven Gärungsstoffwechsel zu betreiben. Nach Wiederherstellung anaerober Kulturbedingungen kann aber erneutes Wachstum erfolgen (O'BRIEN und MORRIS, 1971).

Neuere Arbeiten zeigten, dass *C. acetobutylicum* in der Lage ist gelösten Sauerstoff aus dem Medium zu entfernen und sogar mikroaerob wachsen kann, solange die Rate der O_2-Aufnahme die der kontinuierlichen Zufuhr übersteigt (KAWASAKI *et al.*, 2004). Insgesamt legten diese Erkenntnisse die Idee nahe, dass *C. acetobutylicum*

gelegentlichen Begegnungen mit O_2 keinesfalls völlig hilflos gegenüber steht. Tatsächlich konnten sowohl in Gegenwart von O_2 als auch nach Zugabe von H_2O_2 die verstärkte Expression oxidativer Stressproteine nachgewiesen werden (KAWASAKI *et al.*, 2004; MAY *et al.*, 2004; KAWASAKI *et al.*, 2005). Eine besonders starke Expression bei oxidativem Stress zeigten dabei die das Hitzeschockprotein 21 (Hsp21) kodierenden Zwillingsgene *rbr3* und *rbr3B* (MAY *et al.*, 2004, HILLMANN, 2005; **Abb. 4**).

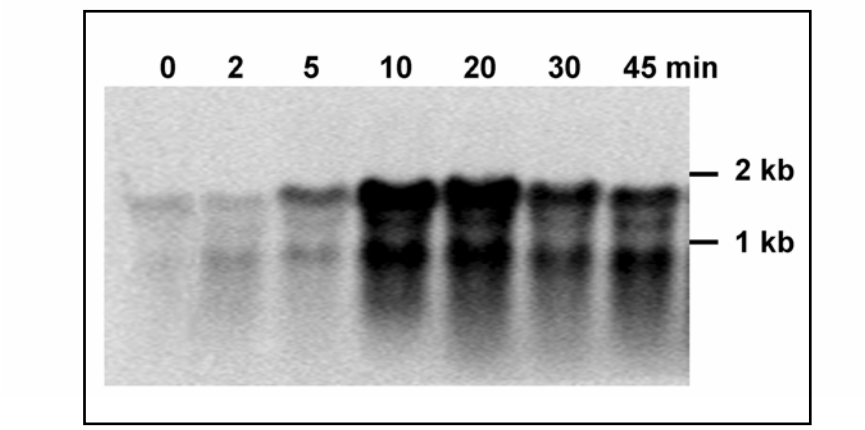

Abb. 4: Expression reverser Rubrerythrin-Gene aus *C. acetobutylicum* unter O_2 (MAY *et al.*, 2004)

Eine *rbr3A* spezifische DNA-Sonde wurde mit RNA (5 µg pro Spur) aus aerob inkubierten Zellen von *C. acetobutylicum* hybridisiert. Zahlen oberhalb der Spuren kennzeichnen die Inkubationszeit der Zellen unter O_2.

Das Hsp21 war zunächst durch schnelle Induktion nach einem Hitzeschock von 30 °C auf 42 °C aufgefallen (BAHL *et al.*, 1995). Es wurde später als Rubrerythrin mit einer reverser Domänenstruktur identifiziert (MAY *et al.*, 2004). Im Gegensatz zu den zuvor beschriebenen Rubrerythrinen, befindet sich beim Hsp21 die typische Rubredoxin-Domäne mit einfachem Fe-S-Cluster nicht am C-terminalen Ende des Proteins, sondern am N-Terminus. Die zweite Domäne ist Ferritin-ähnlich und ist durch ein von einem 4-Helix-Bündel umgebenen, schwefelfreien, 2-Fe-Zentrum charakterisiert (DeMARÉ *et al.*, 1996, **Abb. 5**).

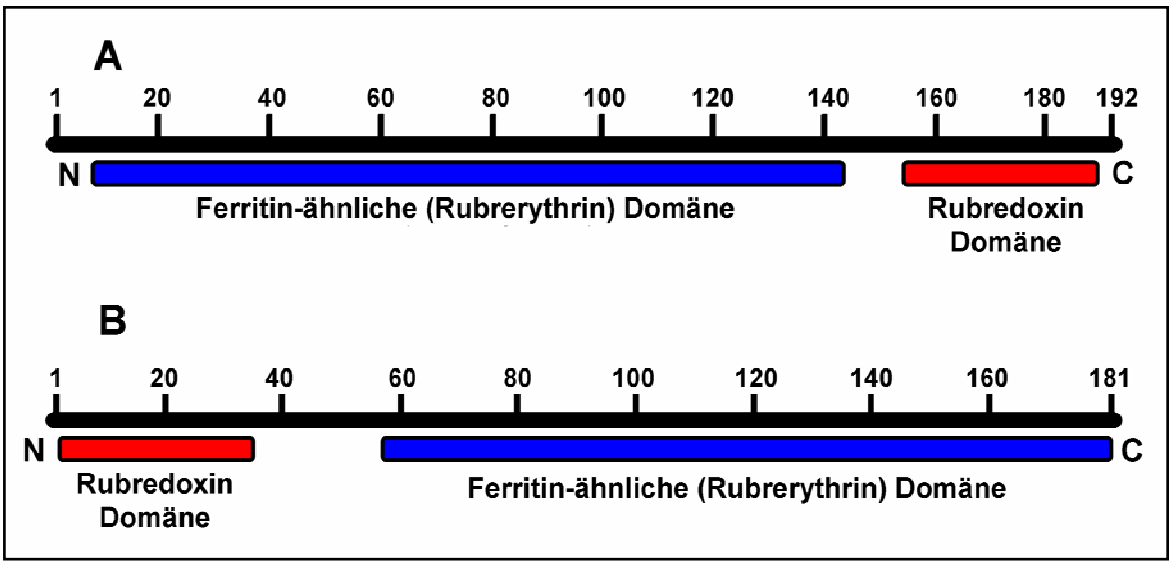

Abb. 5: Domänenstruktur „normaler"- (A) und „reverser" Ruberythrine (B) (MAY *et al.*, 2004)
Zahlen kennzeichnen die Anzahl der Aminosäuren der Proteine.

Normale Rubrerythrine sind erst kürzlich als Rubredoxin-abhängige Peroxidasen in verschiedenen Anaeroben wie *D. vulgaris* und *P. furiosus* beschrieben worden (LUMPPIO *et al.*, 2001; WEINBERG *et al.*, 2004). Auch in *C. acetobutylicum* sind zwei weitere, normale Rubrerythrine kodiert, für die allerdings keine verstärkte Expression bei oxidativem Stress festgestellt werden konnte (MAY *et al.*, 2004; HILLMANN, 2005). Ein ähnliches Bild ergab sich auch für die normalen Rubrerythrine in *C. perfringens* (GEISSMANN *et al.*, 1999; JEAN *et al.*, 2004). Die Situation der vertauschten Domänen bei Rubrerythrinen im gleichen Organismus, sowie ihre differentielle Expression bei oxidativem Stress provozierten Fragen nach der Funktion dieser Proteine (RIEBE *et al.*, 2008) und der Regulation der oxidativen Stressantwort in Anaeroben. Letztere ist bisher kaum untersucht worden und bildete die zentrale Fragestellung der vorliegenden Arbeit.

2 ZIELE DER ARBEIT

Anaerobe Mikroorganismen besitzen häufig die Fähigkeit kurze, periodische Expositionen zu atmosphärischem O_2 nicht nur zu überleben, sondern verfügen auch über eine aktive Verteidigung gegenüber dem für sie toxischen O_2. Diese unterscheidet sich jedoch weitgehend von der oxidativen Stressantwort aerober Organismen. Einige neue Enzyme zur Detoxifikation reaktiver Sauerstoffspezies (ROS) sind erst vor kurzem identifiziert und charakterisiert worden (JENNEY *et al.*, 1999, LUMPPIO *et al.*, 2001; WEINBERG *et al.*, 2004; RIEBE *et al.*, 2007 und 2008). Die Regulation dieser Proteine, als auch der für sie kodierenden Gene, war zu Beginn dieser Arbeit unbekannt. Im obligat anaeroben Bakterium *C. acetobutylicum* ist die Transkription der beiden Gene (*rbr3A* und *rbr3B*) des zunächst als Hitzeschockprotein identifizierten Hsp21 in Gegenwart von O_2 und H_2O_2 stark induziert (MAY *et al.*, 2004; HILLMANN, 2005). Dies lässt nicht nur eine zentrale Funktion dieses Rubrerythrin-ähnlichen Proteins in Gegenwart von O_2 vermuten, sondern legt auch nahe, dass die O_2-abhängige Genexpression in Anaeroben einer differenzierten Regulation unterliegt. Ergebnisse zur O_2-abhängigen Genregulation in *C. acetobutylicum* könnten zudem entscheidend zu einem besseren Verständnis der molekularen Grundlagen obligater Anaerobiose beitragen.

Im Einzelnen sollte die Arbeit daher zur Klärung folgender Fragestellungen beitragen:

1. ***Wie ist die Expression des rbr3-Operons und anderer oxidativer Stressgene reguliert?***

Die erhöhte Expression von *rbr3A/B* nach einem Hitzeschock, sowie nach oxidativem Stress ist bereits nachgewiesen worden. Gibt es außer diesen noch weitere äußere Stressfaktoren die zu einer erhöhten Transkription des Operons führen? Können in der Promotorregion des *rbr3* Operons regulatorische Elemente in der DNA-Sequenz identifiziert werden? Diese wären die Basis zur Identifikation eines Regulatorproteins.

2. ***Wie nehmen anaerobe Organismen O_2 in ihrer Umgebung wahr und wie überlebt C. acetobutylicum in der Gegenwart von O_2?***

Von besonderem Interesse ist, welcher Regulator für die sehr schnell induzierte Transkription des *rbr3* Operons und anderer oxidativer Stressgene verantwortlich ist und ob dieser gleichzeitig in der Lage ist, als direkter oder indirekter Sensor für O_2 zu dienen.

3. Welche Proteine sind maßgeblich am O_2-Verbrauch beteiligt?

Die am O_2-Konsum beteiligten Enzyme sind bisher unbekannt und typische NADH/O_2-Oxidoreduktasen sind bisher nicht identifiziert worden (KAWASAKI *et al.*, 2005). Es ist daher zu vermuten, dass mehrere Komponenten, ähnlich dem ROS-Detoxifikationssystem anaerober Mikroorganismen (**Abb. 3**), an der Reduktion von O_2 beteiligt sind.

4. Gibt es darüber hinaus weitere Anpassungsmechanismen an mikroaerobe Habitate?

Die Fähigkeit des O_2-Verbrauchs, sowie die Existenz einer aktiven Entgiftung reaktiver Sauerstoffspezies lässt es möglich erscheinen, dass *C. acetobutylicum* neben der Beseitigung der „Schadensquellen“ noch weitere Mechanismen entwickelt hat, um O_2 labile Reaktionen des Energiestoffwechsels zu umgehen oder Schäden an essentiellen Zellkomponenten zu minimieren.

3 ERGEBNISSE

Die Ergebnisse werden anhand von 4 Originalpublikationen in englischer Sprache präsentiert. Drei Publikationen sind bereits gedruckt worden, eine weitere ist in Vorbereitung.

3.1 **Hillmann, F.**, R.-J. Fischer und H. Bahl. 2006. The rubrerythrin-like protein Hsp21 of *Clostridium acetobutylicum* is a general stress protein. *Arch. Microbiol.* **185**:270-276.

3.2 **Hillmann, F.**, R.-J. Fischer, F. Saint-Prix, L. Girbal und H. Bahl. 2008. PerR acts as a switch for oxygen tolerance in the strict anaerobe *Clostridium acetobutylicum. Mol. Microbiol.* **68**:848-860.

kommentiert in: Imlay, J. A. 2008. How obligatory is anaerobiosis? *Mol. Microbiol.* **68**:801-804.

3.3 **Hillmann F.**, O. Riebe, R.-J. Fischer, J. D. Caranto, A. Mot, D. M. Kurtz Jr. und H. Bahl. 2009. The flavodiiron proteins FprA1 and FprA2 of *Clostridium acetobutylicum* are rubredoxin dependent O_2 reductases. *FEBS Lett*. **583**: 241-245.

3.4 **Hillmann F.**, C. Döring, A. Ehrenreich, R.-J. Fischer und H. Bahl. 2009. The role of PerR in O_2 affected gene expression of *Clostridium acetobutylicum. Manuskript in Vorbereitung*

Den folgenden Artikeln sind kurze Zusammenfassungen in deutscher Sprache vorangestellt.

3.1 The rubrerythrin-like protein Hsp21 of *Clostridium acetobutylicum* is a general stress protein

Falk Hillmann, Ralf-Jörg Fischer und Hubert Bahl

Archives of Microbiology **185**: 270-276.

Zunächst wurde mit Fluoreszenzfarbstoffen mikroskopisch sichtbar gemacht, dass anhaltende Durchgasung mit Luft nicht unmittelbar eine letale Schädigung der Zellmembran zur Folge hat. Dieser Effekt konnte erst bei starkem oxidativen Stress, simuliert durch H_2O_2, beobachtet werden (Fig. 1). Auch andere Stressfaktoren werden von Bakterien bereits an der Membran wahrgenommen und wirken als Auslöser der „envelope stress response“. Die beiden das Hsp21-kodierenden Zwillingsgene *rbr3A* und *rbr3B* wurden nicht nur nach einem Hitzeschock und oxidativem Stress (O_2 und H_2O_2), sondern auch in Gegenwart weiterer Stressfaktoren verstärkt transkribiert. Tatsächlich konnten durch Northern Hybridisierungen Kälte, NaCl, ein erhöhter pH-Wert und Butanol als Induktoren der Transkription des *rbr3* Operons identifiziert werden (Fig. 2 und 3). Die Gene *rbr3A* und *rbr3B* können deshalb als generelle Stressgene angesehen werden.

Durch „Primer-Extension“ Analysen wurde das 5’-Ende der mRNA in einer Entfernung von 296 Nukleotiden vom Translationsstartpunkt von *rbr3A* bestimmt und ermöglichte die Ableitung möglicher Promotorstrukturen in der -35 und -10 Region (Fig. 4 und 5). Die Nukleotide in der -35 und -10 Region wurden als TTATCA bzw. AACTTT bestimmt - Sequenzen die insbesondere in der -10 Region deutliche Abweichungen vom Consensus-Promotor des vegetativen σ^{70}-Faktor aufwiesen (TTGACA-TATAAT, GRAVES und RABINOWITZ, 1986). Es konnte daher die Regulation über einen alternativen σ-Faktor nicht ausgeschlossen werden. Zusätzlich konnte ein AT-reiches Sequenzpalindrom in der 5’UTR (untranslatierte Region) des Operons als mögliche Bindestelle für ein Regulatorprotein identifiziert werden (Fig. 5).

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ORIGINAL PAPER

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The rubrerythrin-like protein Hsp21 of *Clostridium acetobutylicum* is a general stress protein

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Abstract The small heat shock protein Hsp21 of *Clostridium acetobutylicum* was recently identified as a rubrerythrin-like protein with a rubredoxin-like FeS_4 domain at the N-terminus and a ferritin-like diiron domain at the C-terminus. Here, we report that the two identical tandem genes *rbr3A* and *rbr3B*, which encode the heat shock protein Hsp21, show the transcription pattern of general stress genes. Northern blot analysis indicated that the transcription of the *rbr3AB* operon is induced by various environmental stress conditions: in addition to heat and oxidative stress, an increase of the pH of the growth medium from 4.5 to 6.2, addition of the salt NaCl (400 mM) or of the solvent butanol (3.5% v/v), and lowering the incubation temperature from 37 to 25°C resulted in transiently increased transcript levels. The promoter region deduced from the 5′ end of the mRNA has only limited similarity to the consensus promoter sequence of Gram-positive bacteria. A conserved inverted repeat between this promoter and the initiation codon is proposed to have a regulatory role. Although *C. acetobutylicum* is regarded as a strictly anaerobic bacterium, live/dead staining demonstrated that it can survive exposure to air or H_2O_2 and other stressors to various extents.

Keywords General stress · Heat shock · Anaerobic · *Clostridium* · Flavoprotein

Introduction

The strictly anaerobic, Gram-positive spore former *Clostridium acetobutylicum* is able to change its fermentation metabolism according to growth conditions. During the exponential growth phase sugars are fermented to acetate, butyrate, H_2, and CO_2. While entering the stationary phase *C. acetobutylicum* initiates sporulation and performs a metabolic switch resulting in a conversion of residual sugar and of previously produced acids to butanol and acetone (for a review see Dürre and Bahl 1996). This fermentation was the basis for an industrial solvent production for a long period of time.

In continuous culture experiments several parameters like low pH, threshold concentration of butyrate and growth-limiting factors affecting product formation by *C. acetobutylicum* have been determined (Bahl et al. 1982; Bahl and Gottschalk 1984; Bahl et al. 1986). In general, it can be concluded that solvent formation is favored by stressful growth conditions. Thus, it was not surprising that the induction of heat shock proteins was recorded during the onset of solvent formation (Pich et al. 1990). This observation was recently confirmed by a genomic-scale analysis of the transcriptional program of early sporulation and stationary-phase events in *C. acetobutylicum* (Alsaker and Papoutsakis 2005)

In a previous study, we reported that the small heat shock protein Hsp21 is a rubrerythrin-like protein with reversed domain architecture compared to normal rubrerythrins, i.e., a rubredoxin-like FeS_4 domain at the N-terminus and a ferritin-like diiron domain at the C-terminus (May et al. 2004). Based on their peroxidase (Coulter et al. 1999) and ferroxidase function in vitro (Bonomi et al. 1996) normal rubrerythrins are predicted to be part of an alternative oxygen detoxification system in strict anaerobic bacteria and archea (Lumppio et al. 2001; Weinberg et al. 2004). In agreement with this assumption, increased levels of Hsp21-encoding mRNA are present in *C. acetobutylicum* after an oxidative stress caused by air or H_2O_2 (May et al. 2004; Kawasaki et al. 2004).

A total of four genes encoding rubrerythrins are present in the genome of *C. acetobutylicum* (Nölling et al. 2001). Two normal rubrerythrin genes are positioned at distant sites and are referred to as *rbr1* and

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rbr2. Hsp21, a reversed rubrerythrin, is encoded by two identical tandem genes designated *rbr3A* and *rbr3B*. In this study, we were able to show that the induction of Hsp21 at the transcriptional level is not only triggered by heat and oxidative stress but also by various other stress factors such as salt, increased pH, high concentration of solvents or by a cold shock. Thus, the reversed rubrerythrin Hsp21 can be regarded as a general stress protein. Interestingly, induction of normal rubrerythrins was not observed under the conditions analyzed.

Material and methods

Bacterial strain and growth conditions

Clostridium acetobutylicum ATCC 824 was cultivated routinely in a phosphate limited (0.5 mM) chemostat as described previously (Pich et al. 1990). From this standardized culture 50-ml samples were transferred to 100-ml serum flasks and incubated anaerobically under different stress conditions. A solvent shock was applied by increasing the concentration of butanol up to a final concentration of 382 mM (3.5% v/v). A salt shock was simulated by addition of 200–400 mM NaCl and for an alkaline shock the pH was raised from 4.5 to 6.2 by addition of 5 M NaOH. Incubation under acid stress was achieved by lowering the pH to 3.5, 2.5, and 1.5 with 5 M HCl or 5 M H_2SO_4. For simulation of a cold shock, 10-ml aliquots from the chemostat (incubation temperature of 37°C) were transferred into Hungate-tubes and incubated at 25°C. Other stress experiments were performed as described (May et al. 2004). Samples for RNA isolation and fluorescence imaging were taken before and at different points of time after stress-application.

Fluorescence imaging

Bacterial viability was assessed through use of the LIVE/DEAD® *Bac*Light Bacterial Viability Stain (Molecular Probes, Eugene, Oregon). Three microliters of a solution containing a 1:1 ratio of SYTO-9 to propidium iodide were added to 180 μl of sterile water and 20 μl of culture and incubated at room temperature for 15 min. Aliquots of 6 μl were transferred to Irgalan stained Isopore™-membrane-Filters with 0.2 μm pore-diameter (Millipore, Schwalbach, Germany). Fluorescence images were captured using an Olympus BX51 microscope with appropriate filters (Olympus, Hamburg, Germany). These images were subsequently scored by direct counts of live (green) and dead (red) cells.

RNA isolation and hybridization

Isolation of total RNA with a modified hot-phenol procedure, separation in formaldehyde denaturing gel, and transfer to a nylon membrane were essentially as described previously (Narberhaus and Bahl 1992). The 16S-rDNA-specific probe was labeled by PCR using the Dig DNA Labeling Kit (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. For generation of the 16S-rDNA-specific probe the following oligonucleotides were used: 5′-GTGCCAGCCGCCGCGG-3′ as the forward primer and 5′-CCGTCAATTCATTTAAGTTT-3′ as the reverse primer. For hybridization the same *rbr1*, *rbr2*- and *rbr3A*-specific probes were used as described (May et al. 2004). After blocking, the membranes were incubated with anti-Dig-AP-conjugate. For detection CDP-Star (Roche Diagnostic GmbH, Mannheim, Germany) was used as an ultra-sensitive substrate for alkaline phosphatase according to manufacturer's instructions. Chemiluminescence signals were detected with the Luminescent Image Analyzer LAS-1000 (Raytest, Straubenhardt, Germany). Slot–Blot hybridizations were performed for a relative quantification of mRNA levels and analyzed using the software Image Gauge 3.01 (Fuji, Düsseldorf, Germany). To derive the relative quantity of mRNA levels the chemiluminescence intensity of hybridization signals with RNA from steady state conditions was set as 1 and the signals obtained with RNA from stressed cells were expressed relative to the control.

Primer extension experiments and sequence analysis

Primer extension analysis was performed as described (Kellmann et al. 1990) with IRD800® labeled oligonucleotides PE_rbr3_I with 5′-TTTCCAGCACCACATA CAGGGC-3′ or PE_rbr3_III with 5′-TCTTAGCTGGC AAGCTTTGAGG-3′. Parallel sequencing reactions from the same oligonucleotides were obtained with CycleReader™ Auto DNA Sequencing Kit (Fermentas, St. Leon-Rot, Germany). All signals were analyzed in an automatic sequencer (LI-COR, Bad Homburg, Germany) by using a 6.5% polyacrylamide gel at 1,500 V and 45°C.

Results

Survival of *C. acetobutylicum* under stress

Clostridium acetobutylicum is regarded as a strictly anaerobic microorganism. To demonstrate the effect of oxidative stress on the viability, a live/dead staining was performed before and after exposure of the cells to air or H_2O_2 (Fig. 1). Exposure of the cells to air for 15 min resulted in 9% dead cells, whereas the same incubation time in the presence of H_2O_2 caused 48% dead cells. Aeration of *C. acetobutylicum* for 1 h increased the proportion of dead cells to 18%, but nevertheless demonstrated that this anaerobe can survive this kind of oxidative stress for quite some time and confirmed the earlier observation of O'Brien and Morris (1971) on the

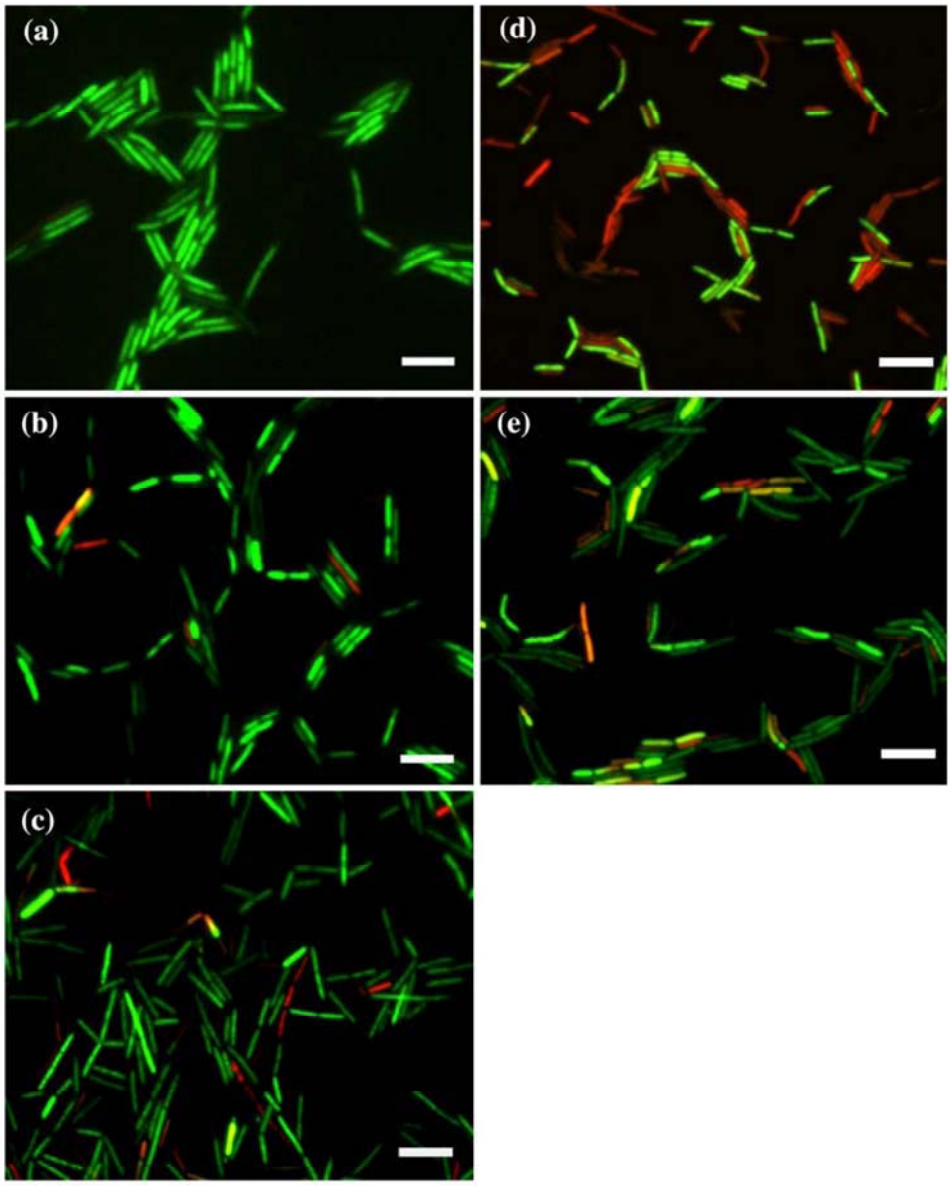

Fig. 1 Live (*green*) and dead (*red*) cells of *C. acetobutylicum* before incubation under stress conditions (**a**), after aeration with compressed air for 15 min (**b**) or 60 min (**c**), 15 min after an addition of 1.5 mM H_2O_2 (**d**) or 400 mM NaCl (**e**). *White bars* indicate 5 μm

behavior of *C. acetobutylicum* towards oxygen. Exposure to other stressors also resulted in a decrease in the viability of *C. acetobutylicum* cells, depending on the extent and the duration of the applied stress. Thus, 66% of cells survived 15 min after a salt shock (400 mM NaCl) and 45% remained viable after 30 min. The percentage of dead cells in the culture correlated with the quality of RNA preparations (data not shown).

Exposure of *C. acetobutylicum* cells to different stressors: mRNA analysis of the *rbr3AB* operon

The synthesis rate of Hsp21 in *C. acetobutylicum* is significantly increased after a heat shock (Pich et al. 1990). Later, corresponding levels of mRNA were found under this condition, but also under oxidative stress (May et al. 2004). Since *rbr3A* and *rbr3B* are obviously induced by two different stressors we investigated whether these genes also react to other stress factors and therefore show the transcription pattern of general stress proteins. Slot and/or Northern blots were prepared with RNA of *C. acetobutylicum* isolated before and at different time points after stress-application. The following stress conditions were analyzed: cold shock from 37 to 25°C, addition of NaCl to a concentration of 400 mM (salt shock), addition of butanol to a final concentration of 382 mM, and an acid (pH from 4.5 to 3.5, 2.5, or 1.5) or alkali (pH 4.5–6.2) shock by the addition of H_2SO_4 (or HCl) or NaOH, respectively. With the exception of acid shock (data not shown) all stressors resulted in a two- to fivefold increase in the transcript levels as revealed by

quantitative analysis of slot–blots with an *rbr3A*-specific probe (Fig. 2). Northern blot analysis with RNA from cold, alkali, and butanol shocked cells identified a 1.6-kb transcript in each case, representing the bicistronic *rbr3AB* operon. The increase in the *rbr3AB* mRNA level was transient with a maximum 10 min after the shock, except for the cold shock where a later maximum (30 min) was observed, which might be due to a general reduction of transcriptional activity at lower temperatures. The degradation of the *rbr3A*-specific mRNA visible as a smear on the Northern blot (Fig. 3c) correlates with the high number of dead cells under these conditions (data not shown).

Slot–blots performed with RNA isolated from stressed cells (see above) and probes against *rbr1* or *rbr2* (encoding normal rubrerythrins) did not reveal increased transcript levels of these genes under the applied stress. Furthermore, the basic transcript levels were lower than those of the reversed *rbr* genes as judged by hardly detectable hybridization signal (data not shown).

Determination of the transcription start point of the *rbr3AB* operon

The transcription start point or more precisely the 5′ end of mRNA of the *rbr3AB* operon was determined by primer extension analysis (Fig. 4) using different primers (PE_rbr3_I; PE_rbr3_III) complementary to the identical 5′ end of *rbr3A* and *rbr3B* or to the 5′ untranslated region (see Fig. 5). Total RNA used in these experiments was isolated before and 2 min after a heat shock from 37 to 42°C or before and at different time points after addition of 200 μM of H_2O_2. Strong signals were obtained with RNA preparations from shocked cells. The primer extension analysis confirmed the fast induction of the *rbr3AB* operon after heat shock (2 min) (May et al. 2004), whereas, the strongest signal after oxidative stress appeared after 10 min. A transcription start point was located 296 bases upstream of the ATG start codon of *rbr3A* with a G as the 5′ end of the mRNA (Fig. 5). The deduced promoter showed only limited similarity to the consensus promoter of σ^{46} containing RNA polymerase of *C. acetobutylicum*. From the data presented in Fig. 4 it is obvious that the identical transcription start point is used under normal and under different stress conditions. A weaker signal representing a several hundred bases longer transcript and obtained with a primer directed against the 5′ end of *rbr3A* (PE_rbr3_I, Fig. 5) is very likely the result of a reverse transcription from the identical coding sequence in *rbr3B* (data not shown). In agreement with this assumption primer extension from PE_rbr3_III (directed against a region upstream of the proposed transcription start point, Fig. 5) resulted in no signal. Thus, Northern blot and primer extension analysis support the view that *rbr3A* and *rbr3B* are organized in a bicistronic operon. Since no other open reading frames are present in the near neighborhood on the same DNA strand these two genes can be regarded as isolated genes.

Computational analysis of the unusually long region between the transcription and translation start points identified a nearly perfect inverted repeat 100 bases upstream of the 5′ end of the *rbr3A* gene (Fig. 5, IR1). The formation of a hairpin structure of the mRNA in vivo is unlikely due to the low free energy of −0.6 kcal/mol at 37°C (Zuker 2003). Thus, this sequence might be a DNA binding site for a potential regulator. Interestingly, this sequence is conserved in front of various genes whose products are proposed to be involved in iron homeostasis or in defense against oxidative stress (data not shown).

Discussion

In bacteria a change of growth conditions leads to an altered expression of various proteins. This is especially the case under stressful conditions when the growth rate is affected or even the damage of cellular components is imminent (for an overview see Storz and Hengge-Aronis 2000). Some of the proteins are only induced by one particular stress condition, others respond to various stress factors. Here, we were able to show that the

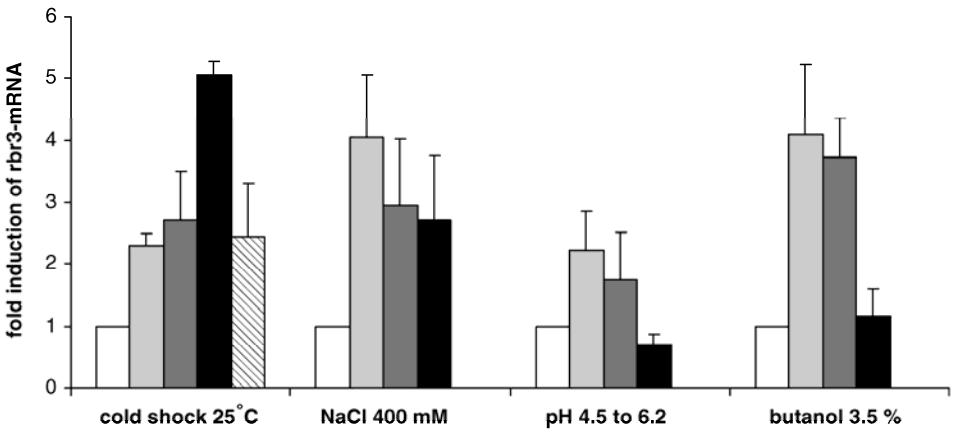

Fig. 2 Level of *rbr3*-mRNA under various stress conditions. Cells of *C. acetobutylicum* were taken from the continuous culture and were either submitted to a cold shock from 37 to 25°C, a NaCl-treatment of 400 mM, an increase in pH from 4.5 to 6.2, or an increased concentration of butanol. Aliquots for RNA isolation were harvested before stress was applied (*white bars*), and after 5 min (*light gray bars*), 10 min (*dark gray bars*), 30 min (*black bars*), and 60 min (*shaded bars*), respectively

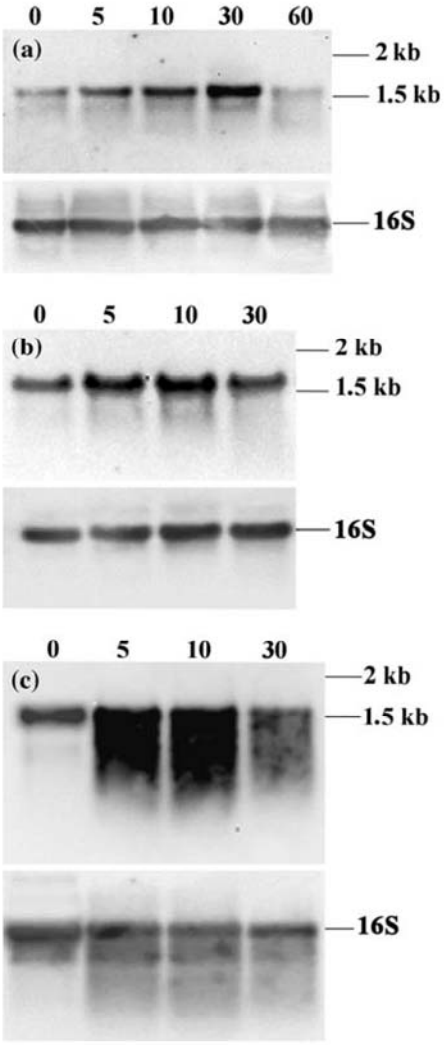

Fig. 3 Northern blot analysis of total RNA from *C. acetobutylicum* isolated before and after a cold shock (**a**) an alkaline stress (**b**) or an addition of butanol to the growth medium (**c**). Each lane contains 5 μg of RNA and was probed with a Dig-labeled fragment of *rbr3A*. Below each lane a hybridization signal with a 16S RNA-specific probe is shown as a control. *Numbers above each lane* indicate the time in minutes after incubation under stress

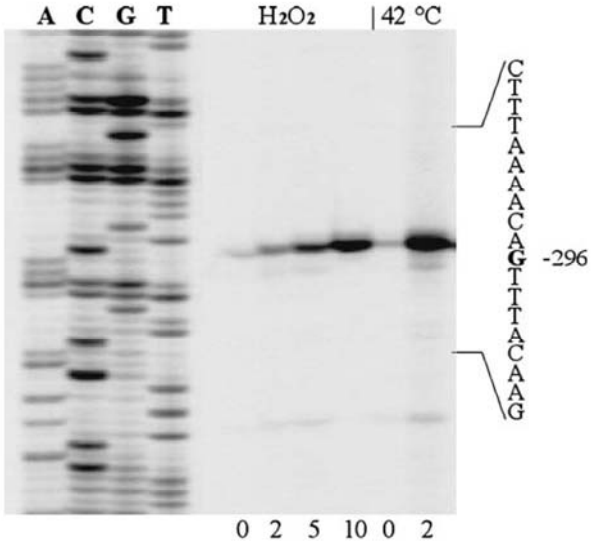

Fig. 4 Mapping of the 5′ end of mRNA of the *rbr3AB* operon by primer extension analysis. An IRD800® labeled oligonucleotide complementary to the 5′ end of *rbr3A* was hybridized to 10 μg each of total RNA from *C. acetobutylicum* isolated before (0) and after a heat shock or H_2O_2-treatment. *Numbers below each lane* indicate the incubation time in minutes under stress conditions. The primer extension products were analyzed on a sequence gel. A, C, G, and T are products of the sequencing reaction obtained by using the same oligonucleotide as a primer. The depicted sequence represents the antisense strand. A *bold G* marks the transcription start-site of the *rbr3*-mRNA respective to the *rbr3A* start codon

induction of Hsp21 is triggered by a wide range of different environmental signals like heat, oxidative and alkaline stress, or increased concentration of salt and solvents. Therefore, Hsp21 belongs to the group of proteins which are part of multiple stimulons. The majority of analyzed stress signals caused an increased amount of mRNA within the first 5–10 min of the shock. During the next 30–60 min the transcript levels decline to a new steady state level which approaches the amount present under normal growth conditions. Our data suggest that Hsp21, originally identified as a heat shock protein, might have an in vivo function which is linked to or even promotes the general adaptation of the cells to environmental pressure.

In addition to heat, oxidative stress seems to be one of the stress situations where the function of Hsp21 is needed. Previously, Hsp21 in *C. acetobutylicum* was identified as a reverse rubrerythrin (May et al. 2004), with the rubredoxin domain ($Fe[SCys]_4$) at the N-terminus and a C-terminal ferritin-like domain with an oxo-bridged diiron site (deMaré et al. 1996). Whether the function of these rubrerythrins differs from that of normal Rbrs has not yet been established. So far, only the latter ones have been characterized as to their in vitro function and were predicted to represent the terminal component of an alternative oxygen detoxification system in strict anaerobic bacteria and archea. However, an increase in the transcript levels of the corresponding *rbr* genes as a response to oxidative stress has not been observed in all organisms studied. In *Porphyromonas gingivalis* a two- to threefold increase of mRNA levels was found after 2 h incubation under oxidative stress (oxygen or H_2O_2, Sztukowska et al. 2002). In contrast, oxidative stress did not influence *rbr* expression in *Clostridium perfringens* (Geissmann et al. 1999) or even was responsible for a down regulation in *Desulfovibrio vulgaris* Hildenborough (Fournier et al. 2006). Here, we demonstrate that the reverse *rbrs* of *C. acetobutylicum* respond to several stressors and show the transcription pattern of general stress genes. In contrast, induction of the two normal *rbr* genes could not be observed under the conditions analyzed. The fact that genes for both types of Rbr proteins are present in *C. acetobutylicum*

Fig. 5 The promoter region of the *rbr3AB* operon. Only the antisense strand is shown. *Arrows* below the lines indicate the positions of the oligonucleotides used for primer extension. The potential ribosome binding site is *underlined*. The start codon of *rbr3A*, the transcription start-site, and the corresponding −35 and −10 regions are marked by *bold letters*. *Arrowheads* above the DNA sequence show a region of dyad symmetry

and show different transcription patterns is pointing towards different regulation and also different function. The presence of both, normal and reversed, *rbr* genes is conserved in the genomes of all completely sequenced clostridia (*C. acetobutylicum*, *C. perfringens*, and *C. tetani*) and in other anaerobic eubacteria (Geer et al. 2002).

In *Bacillus subtilis* and in a number of other Gram-positive bacteria, general stress proteins are under the control of RNA polymerase holoenzyme containing an alternative sigma factor, e.g., σ^B (Chan et al. 1998; Ferreira et al. 2001; Hecker and Völker 2001). So far, a gene for σ^B could not be identified in the genome of *C. acetobutylicum* (Sonenshein et al. 2005). The deduced promoter region of *rbr3A* has only limited sequence similarity to the consensus promoter sequence of *C. acetobutylicum* (Young et al. 1989; Bahl et al. 1995). Thus, it cannot be excluded that the transcription of the *rbr3AB* operon of *C. acetobutylicum* is regulated by an alternate sigma factor whose identity has to be elucidated, i.e., whether the similarity of the extended promoter region to that of many TcdR-dependent genes (GTTTACA-17nt-CTTTTT) in *C. difficile* (Mani et al. 2002) is of biological significance. TcdR represents a family of ECF σ-factors which has so far exclusively been identified in pathogenic clostridia (Moncrief et al. 1997; Mani and Dupuy 2001). Furthermore, a putative role of the conserved inverted repeat (Fig. 5) has to be considered in the analysis of the regulation of this stress operon.

The elucidation of the function of the general stress protein Hsp21 of *C. acetobutylicum* and the regulation of its gene in the network of different stimulons will be an important task, especially since this protein is widespread among anaerobic bacteria.

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3.2 PerR acts as a switch for oxygen tolerance in the strict anaerobe *Clostridium acetobutylicum*

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Im fakultativ aeroben *Bacillus subtilis* bindet der Peroxidregulator spezifisch an AT-reiche DNA Sequenzen und unterdrückt dadurch die Transkription von Genen des Peroxidstimulons (BSAT *et al.*, 1998). Ein ähnliche DNA Sequenz befand sich auch in der 5'UTR des *rbr3* Operons von *C. acetobutylicum* (HILLMANN *et al.*, 2006). In *C. acetobutylicum* und anderen Clostridien konnten Gene für PerR ähnliche Proteine identifiziert werden (Fig. 1 und Table 1). Der Artikel beschreibt die Herstellung (Fig. S1) und Charakterisierung einer *perR*-Deletionsmutante von *C. acetobutylicum*.

Eine der offensichtlichsten und interessantesten Eigenschaften dieser Mutante war ihre deutlich erhöhte Toleranz gegenüber O_2. So war es diesen Kolonien möglich Wachstum selbst in aerober Atmosphäre fortzusetzen (Fig. 2). Genauere Analysen in Flüssigkultur bestätigten, dass diese Mutante auch eine 7-fach erhöhte Resistenz gegenüber H_2O_2 aufwies und in der Lage war über Stunden in aerober Flüssigkultur zu überleben (Fig. 3A und 3B). Darüber hinaus war eine zeitlich limitierte Zunahme der Lebendzellzahl und der optischen Dichte nachweisbar (Fig. 3 und 4). Sowohl der O_2 Verbrauch, als enzymatische Aktivitäten zur Reduktion von ROS waren in der Abwesenheit von PerR 7-14-fach erhöht (Fig. 5, Table 3). In Übereinstimmung mit diesen Aktivitäten kam es in der Mutante zur Überexpression von Komponenten des alternativen Detoxifikationsystems (Fig. 7 und 8). Die am stärksten induzierte Expression in der Mutante betraf ein unbekanntes Flavoprotein (FprA1) und vor allem das Hsp21, dessen rote Farbe bereits im Rohextrakt sichtbar war (Fig. S5). Es konnte deshalb geschlussfolgert werden, dass PerR ein zentraler Transkriptionsrepressor der O_2-Abwehr obligat Anaerober ist und die volle Aktivierung des PerR Regulons ein Überleben in oxidativer Umgebung ermöglicht.

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PerR acts as a switch for oxygen tolerance in the strict anaerobe *Clostridium acetobutylicum*

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Summary

Clostridia belong to those bacteria which are considered as obligate anaerobe, e.g. oxygen is harmful or lethal to these bacteria. Nevertheless, it is known that they can survive limited exposure to air, and often eliminate oxygen or reactive derivatives via NAD(P)H-dependent reduction. This system does apparently contribute to survival after oxidative stress, but is insufficient to establish long-term tolerance of aerobic conditions. Here we show that manipulation of the regulatory mechanism of this defence mechanism can trigger aerotolerance in the obligate anaerobe *Clostridium acetobutylicum*. Deletion of a peroxide repressor (PerR)-homologous protein resulted in prolonged aerotolerance, limited growth under aerobic conditions and rapid consumption of oxygen from an aerobic environment. The mutant strain also revealed higher resistance to H_2O_2 and activities of NADH-dependent scavenging of H_2O_2 and organic peroxides in cell-free extracts increased by at least one order of magnitude. Several genes encoding the putative enzymes were upregulated and identified as members of the clostridial PerR regulon, including the heat shock protein Hsp21, a reverse rubrerythrin which was massively produced and became the most abundant protein in the absence of PerR. This multifunctional protein is proposed to play the crucial role in the oxidative stress defence.

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Introduction

While early studies suggested that obligate anaerobiosis was tightly linked to the absence of catalase and superoxide dismutase (SOD) activities (McCord *et al.*, 1971) and hence, these organisms die as a result of a lack of efficient scavengers, it becomes more and more obvious that molecular oxygen itself is the harmful or lethal agent for these bacteria (Imlay, 2006). More recent approaches suggested that obligate anaerobiosis is tightly linked to the central metabolism of these organisms. For example, pyruvate-ferredoxin-oxidoreductase, a central enzyme in many anaerobic fermentation pathways, is quickly inactivated in the presence of oxygen, apparently as a result of oxidative inactivation of its exposed iron-sulphur sites (Meinecke *et al.*, 1989; Imlay, 2006).

Nevertheless, anaerobic bacteria defend themselves against the deleterious effects of oxygen. Some even reveal SOD activity to provide additional protection against high levels of O_2^- (Brioukhanov *et al.*, 2002). Following their exposure to oxidizing conditions, anaerobes express a number of alternative scavenging enzymes including the components of an alternative detoxification system (Jenney *et al.*, 1999). This system employs superoxide reductases (SOR), peroxidases or oxygen reductases which act as electron carriers from NAD(P)H to O_2^-, H_2O_2 or O_2, and seem to be conserved in anaerobes. Components have been isolated and characterized from a number of organisms, including *Pyrococcus furiosus* (Weinberg *et al.*, 2004), *Desulfovibrio desulphuricans* (Romão *et al.*, 1999), *Desulfovibrio vulgaris* (Wildschut *et al.* 2006), and *Clostridium acetobutylicum* (Riebe *et al.*, 2007).

Clostridium acetobutylicum was an industrial important producer of organic solvents throughout the first half of the last century and regained much attention recently as a source for biofuels from renewable resources (for a review see Bahl *et al.*, 1988; Dürre, 2007). It quickly halts growth and fermentation upon a short exposure to atmospheric oxygen, and resumes both only when anaerobic conditions are nearly restored (O'Brien and Morris, 1971). A recent study revealed that *C. acetobutylicum* was able to continue growth under microoxia, but only when the rate of oxygen consumption exceeded the rate of oxygen

influx (Kawasaki *et al.*, 2004), thereby maintaining an essentially anoxic environment.

This microaerobic survival is thought to be partially due to the anaerobic pathway of detoxification of reactive oxygen species (ROS), as several of the corresponding genes are upregulated, including desulfoferrodoxin (*dfx*), recently characterized as a SOR (Riebe *et al.*, 2007) and two rubrerythrins with reversed domains that may act as NAD(P)H peroxidases [Rbr3A and B (encoded by twin genes)] (May *et al.*, 2004; Kawasaki *et al.*, 2004; 2005). Previous studies on the latter two genes identified the corresponding protein(s) as 'reverse rubrerythrins' and demonstrated that they are induced in response to oxidants and other stressors (May *et al.*, 2004; Hillmann *et al.*, 2006). Besides, a number of other genes, including flavoproteins and other peroxidases, are upregulated under oxidative conditions (Kawasaki *et al.*, 2005), but have not yet been characterized in function. However, little is known about the cellular components and mechanisms involved in the regulation of oxygen-mediated gene expression in anaerobes. In the present study, we analysed whether manipulation of the regulatory mechanisms of their oxidative stress response provides new insights as to what extent anaerobiosis is obligate.

Gram-positive aerobes have evolved efficient mechanisms to sense oxidative stress in their environment. The peroxide repressor PerR from *Bacillus subtilis* is a member of the ferric uptake regulator (Fur) protein family and acts as a transcriptional repressor that senses H_2O_2 by metal-catalysed histidine oxidation (Lee and Helmann, 2006). Mutations in the *perR* homologues of the facultative anaerobes *B. subtilis* (Bsat *et al.*, 1998), *Staphylococcus aureus* (Horsburgh *et al.*, 2001) and the aerotolerant *Streptococcus pyogenes* (King *et al.*, 2000) generated strains with increased resistance to H_2O_2, by derepressing target genes including the ones coding for alkylhydroperoxide reductase, catalase and MrgA, a Dps-like protein providing DNA protection from oxidative damage. Besides catalase, also a gene coding for a Dps homologue is missing in the genome of *C. acetobutylicum* (Nölling *et al.*, 2001). Thus it seemed interesting whether a PerR-homologous protein might be involved in the regulation of the oxidative stress response of an obligate anaerobe. Here we report that a PerR-like protein acts as repressor of proteins involved in the oxygen defence of *C. acetobutylicum*, and that its aerobic survival drastically increased following its full derepression.

Results

Identification of perR-homologous genes in C. acetobutylicum *and other clostridia*

The peroxide repressor PerR from *B. subtilis* was previously identified as a member of the Fur protein family

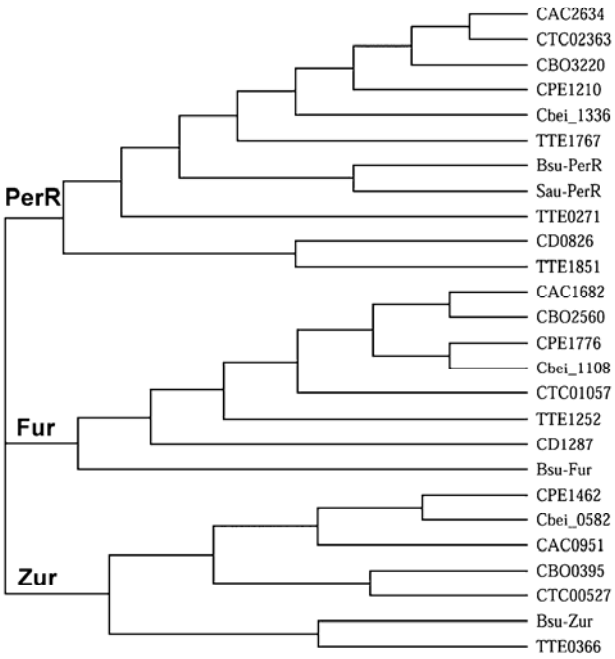

Fig. 1. PerR-, Fur- and Zur-like proteins are conserved among clostridia. A phylogentic tree of Fur-like proteins from selected clostridia and other Gram-positive bacteria was constructed based on a multiple sequence alignment. An un-rooted cladogram was constructed as described in *Experimental procedures*. Fur-like proteins are represented by their open reading frame number, or in combination with the species. Bsu, *Bacillus subtilis*; CAC, *Clostridium acetobutylicum ATCC824*; Cbei, *C. beijerinickii NCIMB 8052*; CBO, *C. botulinum A str. ATCC* 3502; CD, *C. difficile 630*; CPE, *C. perfringens str. 13*; CTC, *C. tetani E88*; TTE, *Thermoanaerobacter tencongensis*.

(Bsat *et al.*, 1998). Other members of this protein family include Fur itself, and the zinc uptake regulator Zur (Fuangthong and Helmann, 2003). Global approaches to identify the corresponding genes of the PerR regulon in *B. subtilis* (Helmann *et al.*, 2003), *Synechocystis* sp. strain PCC 6803 (Li *et al.*, 2004) and *S. pyogenes* (Brenot *et al.*, 2007) have established that this protein acts primarily as a repressor of genes involved in the peroxide stimulon. To identify PerR-homologous proteins in anaerobic organisms, we performed BLAST homology searches with *B. subtilis* PerR and the sequenced genomes of *C. acetobutylicum ATCC824* and other members of the clostridia (Altschul *et al.*, 1997). These data revealed that genes encoding Fur-homologous proteins are well conserved among the annotated genomes from different members of the clostridia. Genes encoding proteins of the Fur family were found in all selected clostridia, including *Clostridium beijerinckii*, the pathogenic *Clostridium difficile*, *Clostridium perfringens*, *Clostridium botulinum*, *Clostridium tetani*, and the more distantly related *Thermoanaerobacter tencongensis*. Fur-, Zur- and PerR-like proteins of these strains all cluster with their *B. subtilis* homologues (Fig. 1). Interestingly, the comparatively

Table 1. Fur-like proteins in *C. acetobutylicum*.

ORF *C. acetobutylicum*/ *B. subtilis* protein	Aminoacid identity/similarity (%)		
	cac0951	*cac1682*	*cac2634*
cac0951	100/100	33/56	27/53
cac1682		100/100	32/56
Fur (*B. subtilis*)	33/50	49/70	34/54
Zur (*B. subtilis*)	28/49	29/55	28/47
PerR (*B. subtilis*)	31/53	32/58	36/61

The deduced aminoacid sequences of open reading frames (ORFs) encoding putative Fur family proteins in the genome of *C. acetobutylicum* were compared with the three known regulators from *B. subtilis*.

small genome of *T. tencongensis* (2.69 Mbp) comprises five independent copies of *fur* family genes, with three of the encoded proteins clustering in the PerR group. For *C. acetobutylicum*, we identified three genes (*cac0951*, *cac1682* and *cac2634*) encoding Fur-like proteins. For the clostridial Fur proteins, CAC1682 and CAC2634, amino acid identities are lower between each other, as compared with their putative homologues (CAC1682 with Fur and CAC2634 with PerR) in *B. subtilis* (Table 1). Furthermore, all amino acids involved in metal binding of *B. subtilis* PerR are only conserved in *cac2634*, the putative PerR homologue of *C. acetobutylicum* (Fig. S1). We therefore anticipated that *cac2634* might play a role in the regulation of the oxidative stress-dependent gene regulation in this anaerobe. To determine its functional role *in vivo*, we constructed a deletion mutant of the clostridial *perR* homologue *cac2634* using a recently established method for homologous recombination in *C. acetobutylicum* (Soucaille *et al.*, 2006; Fig. S2) and generated strain *C. acetobutylicum* Δ*perR*.

Total iron levels are not altered in the absence of PerR

Considering the junction of oxygen and metal homeostasis, anaerobic bacteria in particular are faced with a threatening dilemma: it is well established that a high amount of free intracellular iron, as observed under anaerobic conditions, boosts the production of hydroxyl radicals by Fenton chemistry, and therefore intensifies oxidative stress (Keyer *et al.*, 1995; Keyer and Imlay, 1996). On the other hand, iron is an essential cofactor of the key detoxification enzymes. Furthermore, in *Neisseria gonorrhoea*, PerR-dependent manganese accumulation triggered resistance to superoxide (Wu *et al.*, 2006). Hence, we were interested whether a deletion of *perR*, as a member of the Fur family, affects metal transport in *C. acetobutylicum*. We measured the cellular content of the main trace metals by inductively coupled plasma atomic emission spectroscopy. The total concentration of cellular ^{26}Fe was unaffected by the absence of PerR (14.6 nmol mg^{-1} dry weight in both the wild type and the mutant; Table 2). While only ^{30}Zn built up by more than twofold in *C. acetobutylicum* Δ*perR*, changes in the concentration of all other elements analysed (^{20}Ca, ^{27}Co, ^{29}Cu, ^{19}K, ^{12}Mg, ^{25}Mn and ^{28}Ni) were rather small and might be of low biological significance. In conclusion, the intracellular level of iron, the main catalysing agent in hydroxyl radical production, is regulated by a mechanism independent of PerR. We presume that one of the other Fur homologues, encoded in the genome of *C. acetobutylicum*, e.g. *cac1682*, as the closest *B. subtilis* Fur homologue, is involved in this process. Deletion of the *fur* gene in *Escherichia coli* yielded a phenotype which overimported iron and as a result was highly susceptible to oxidative damage by exogenous H_2O_2 (Keyer and Imlay, 1996). At the same time, the elevated level of zinc may substitute for iron in different oxidative stress proteins, namely thioredoxin or rubrerythrin respectively (Collet *et al.*, 2002; Li *et al.*, 2003). Moreover, elevated zinc uptake as a result of *perR* mutation may help to prevent thiol oxidation (Gaballa and Helmann, 2002), as the antioxidant role of zinc as an antagonist of iron and copper in biological systems has been reviewed (Powell, 2000).

PerR deletion triggers aerotolerance and H_2O_2 resistance in C. acetobutylicum

The obtained mutant, *C. acetobutylicum* Δ*perR*, was viable, but grew more slowly than the wild type in anaerobic minimal medium. The growth rate of the mutant was reduced by up to 25% and cells also displayed an altered morphology under all conditions analysed (Fig. S3 and S4). Cells of the *perR*-deleted strain were more than twofold longer than those of the wild type and often formed chains consisting of two to five individuals (Fig. S4). Colonies of both strains did not differ in size

Table 2. Metals in *C. acetobutylicum*.

Element	Abundance (nmol mg^{-1})		
	perR$^+$	*perR*$^-$	Fold change
^{20}Ca	4.3 ± 0.5	5.1 ± 1.4	1.1
^{27}Co	0.05 ± 0.01	0.04 ± 0.02	0.8
^{29}Cu	0.37 ± 0.13	0.25 ± 0.08	0.7
^{26}Fe	21 ± 6	22 ± 6	1.0
^{19}K	170 ± 32	140 ± 18	0.8
^{12}Mg	54 ± 20	34 ± 12	0.6
^{25}Mn	0.34 ± 0.09	0.29 ± 0.12	0.8
^{28}Ni	0.15 ± 0.06	0.22 ± 0.09	1.5
^{30}Zn	1.5 ± 0.3	3.9 ± 0.7	2.6

Quantities were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Values are expressed as the amount of substance per dry weight of cells. Error bars indicate the standard deviation of the mean value of three independent experiments.

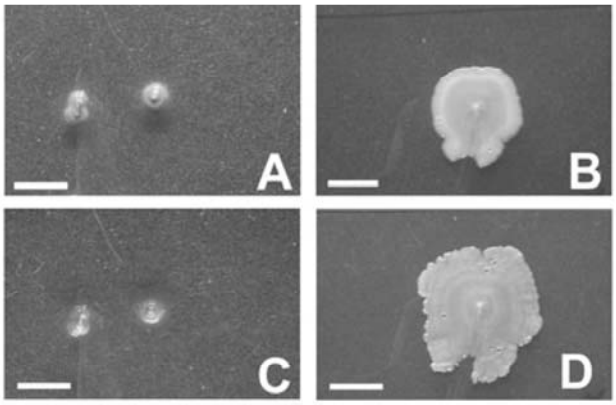

Fig. 2. Growth of *C. acetobutylicum* colonies in ambient air. *C. acetobutylicum perR*$^+$ (A, C) and *perR*$^-$ (B, D) after 10 days (A, B) and 50 days (C, D) of incubation in ambient air at room temperature. Colonies were first grown anaerobically at 35°C for 48 h on solid complex medium and exposed to aerobic conditions. White bars indicate 5 mm.

when incubated under anaerobic conditions for 48 h. When removed from the anaerobic chamber and further incubated under ambient air, *C. acetobutylicum MGC-cac15* ceased growth, while *C. acetobutylicum* Δ*perR* continued to grow for days (Fig. 2).

Therefore, we wondered whether the loss of the putative repressor of PerR-responsive genes would enable a growing culture of *C. acetobutylicum* to better cope with oxidative stress. Mutations in the *perR* homologues of *B. subtilis*, *S. pyogenes* and *S. aureus* generated strains with increased resistance to the highly genotoxic oxidizing agent H_2O_2. Consequently, we tested whether this ability was enhanced in the mutant. Aliquots of cells were incubated with increasing amounts of H_2O_2, and the survival of cells after 30 min of incubation was monitored as colony-forming units (cfu ml^{-1}). The viability of the wild type was severely affected by H_2O_2 exceeding 100 μM, while even a sevenfold higher concentration of H_2O_2 did not drastically reduce the survival of *C. acetobutylicum* Δ*perR* (Fig. 3A). The sensitivity of *C. acetobutylicum* towards H_2O_2 was largely dependent on Fenton chemistry, as described by Imlay and Linn (1988). Thus, addition of an intracellular iron chelator (1 mM dipyridyl) attenuated peroxide-dependent killing of wild-type *C. acetobutylicum* (Fig. 3A). This demonstrates that PerR plays a critical role in the peroxide response of *C. acetobutylicum*, and that even an organism that lacks catalase can withstand considerable amounts of H_2O_2 if free intracellular iron is excluded from the Fenton-based generation of hydroxyl radicals (•OH).

It seemed interesting whether enhanced H_2O_2 protection would also allow this strict anaerobe to better tolerate oxygen. To test oxygen sensitivity in liquid cultures, we transferred anaerobic liquid cultures of the two strains to a well-aerated environment on a rotary shaker and, at regular intervals, samples were drawn to determine the number of survivors. The parental strain immediately ceased growth, and survival declined to less than 0.01% in the first hour of aeration. Even low doses of dipyridyl (0.1 mM) efficiently sequestered free intracellular iron and aerobic killing of the wild type was considerably attenuated (Fig. 3B). Conversely, in the absence of dipyridyl, the *perR*-deleted strain not only retained complete viability for up to 6 h under aerobic conditions, but it actually displayed an increasing population. The number of viable cells increased up to sevenfold during the first 2 h of aeration (Fig. 3B), which was in agreement with increasing optical densities of cultures of the *perR*-deleted strain in presence of oxygen (Fig. 4). As judged by light microscopy, the majority of the mutant cells also maintained their

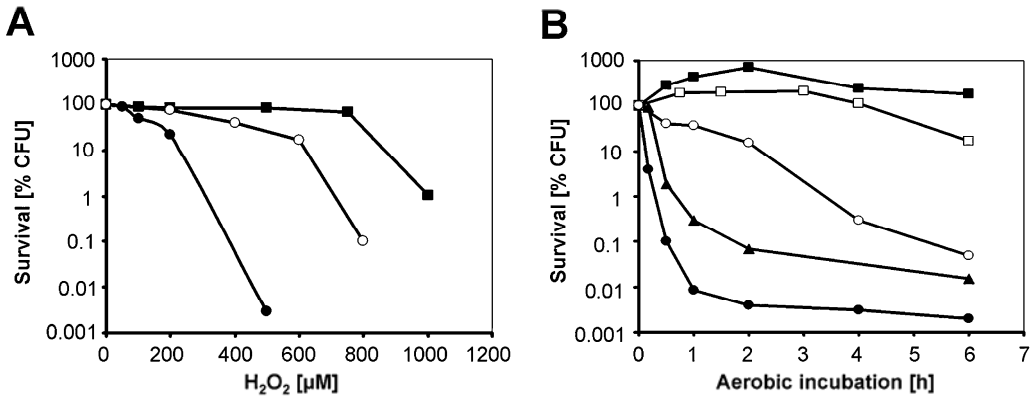

Fig. 3. Survival of *C. acetobutylicum* under oxidative stress. After anaerobic cultivation to an OD_{600} of 0.6, exponentially growing cells of *C. acetobutylicum perR*$^+$ (circles), *C. acetobutylicum perR*$^-$ (squares) or the complemented *C. acetobutylicum* Δ*perR-perR*$^+$ (triangles) were incubated either (A) with different concentrations of H_2O_2 for 30 min, or (B) aerobically at 35°C and 200 r.p.m. on a rotary shaker. Survival of *C. acetobutylicum perR*$^+$ in the presence of the iron chelator dipyridyl, and of *C. acetobutylicum perR*$^-$ at 100-fold lower cell density is indicated by open circles and open squares respectively. Samples were drawn at the indicated time points and the cfu were determined according to a non-stressed control (100%). Data represent the mean value of three independent experiments.

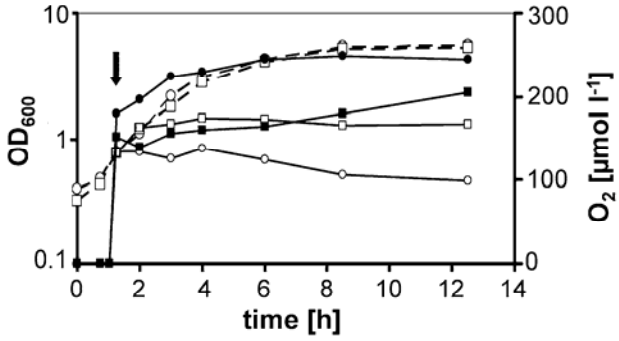

Fig. 4. Optical density and O_2 concentration during aeration. The optical density of cultures of *C. acetobutylicum perR*$^+$ (open circles) and *C. acetobutylicum perR*$^-$ (open squares) was followed during growth in CGM. Both strains were initially grown anaerobically. A black arrow indicates when cultures were shaken in ambient air at 200 r.p.m. (solid lines) or left anaerobically (dashed lines). Closed symbols with solid lines (*perR*$^+$, circles; *perR*$^-$, squares) indicate the O_2 concentration in shaken cultures of both strains.

motility for several hours in the presence of air, while the wild type became immotile very shortly after aeration. The extended aerobic survival of *C. acetobutylicum* Δ*perR* was not dependent on high cell densities as 100-fold dilutions of the cells maintained similar viability (Fig. 3B), indicating that survival was not simply based on the re-establishment of anaerobiosis in the growth medium. Following expression of a plasmid-encoded *perR* from its own promoter in *C. acetobutylicum* Δ*perR*, wild-type oxygen sensitivity was nearly restored (Fig. 3B).

Oxygen and ROS scavenging are controlled by PerR

Although altered iron disposition might largely prevent deleterious effects of ROS during oxygen exposure, enhanced expression of detoxification enzymes would be beneficial by providing an additional protection. As it has previously been shown that anaerobic bacteria are able to reduce ROS and also use oxygen reduction to create an anaerobic environment, we anticipated that these activities contribute to aerobic survival of *C. acetobutylicum* Δ*perR*. Hence, we determined the oxygen consumption and enzyme activities for ROS-dependent oxidoreductases for both strains. For the determination of oxygen consumption, an O_2 optode was used as described in the *Experimental procedures* and according to Warkentin *et al.* (2007). Cells of the parental strain and the mutant were grown in clostridial growth medium (CGM) to the mid-exponential growth phase and aerated with compressed air for at least 5 min. This time period was found to be sufficient to obtain the maximum oxygen saturation of the medium of ~260 µmol l^{-1} O_2. Immediately afterwards, oxygen influx was stopped and the O_2 concentration was monitored over time. *C. acetobutylicum* consumed oxygen at a maximum rate of 0.5 fmol h^{-1} cell^{-1} (Fig. 5A). The rate decreased to nearly zero in the following 80 min (Fig. 5B), corresponding to the increasing number of dead cells as a result of oxygen exposure (Fig. 3B, *perR*$^+$). At this point, a second aeration of the cells did not reconstitute any activity, as oxygen saturation was essentially maintained (data not shown). In comparison with the parental strain, the maximum oxygen consumption rate was nearly 30-fold higher in the *C. acetobutylicum* Δ*perR* (16 fmol h^{-1} cell^{-1}, *perR*$^-$), leading to a completely reduced environment in less than 15 min after the addition of air (Fig. 5B) and depending on cell density, sufficient to considerably reduce the O_2 concentration in a shaken culture (Fig. 4).

In anaerobic bacteria, aeration is thought to especially boost the production of ROS, e.g. H_2O_2 or superoxide, as a result of their low-potential electron transfer enzymes. Consequently, we further analysed whether the ability to scavenge these molecules was also increased in the mutant. To determine the respective enzyme activities (Table 2), mid-exponential cells of both strains were harvested and used as a source of anaerobic protein.

Using H_2O_2 and t-butylhydroperoxide as substrates for peroxidases, increased peroxide reductase activities by a factor of 10–14 were observed in the absence of their repressor. When comparing both strains, an at least 14-fold induction of NADH oxidase in the *perR*-deleted *C. acetobutylicum* strain was detected, revealing the highest of all reduction activities determined in this study

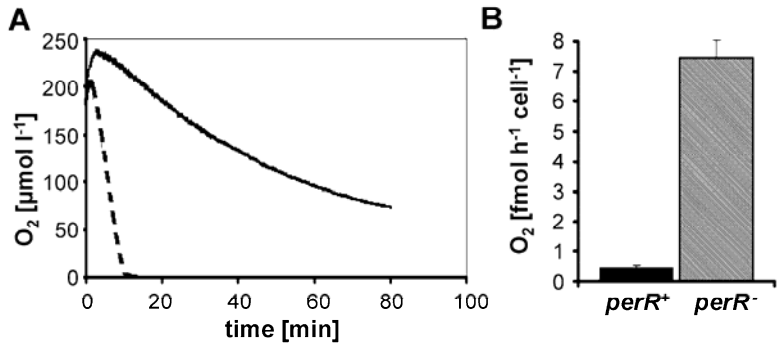

Fig. 5. O_2 consumption of *C. acetobutylicum*
A. O_2 concentration at 37°C in mid-exponential cultures of *C. acetobutylicum perR*$^+$ (solid line) and *perR*$^-$ (dashed line) was monitored following a single aeration with atmospheric air.
B. Maximum O_2 consumption rates of cells from both strains at 37°C, calculated from the maximum decrease in the O_2 concentration. Error bars indicate the standard deviation of the mean value of three independent experiments.

Table 3. Enzyme activities for the scavenging of oxygen or ROS in wild-type *C. acetobutylicum* (*perR*$^+$) and the *perR*-deleted strain (*perR*$^-$).

Enzyme	Activity (mU mg^{-1} of protein)		
	perR$^+$	*perR*$^-$	Induction fold
NADH oxidase	31 ± 4	430 ± 60	14.1
NADPH oxidase	14 ± 3	65 ± 9	4.6
NADH–H_2O_2-oxidoreductase	17 ± 2	190 ± 20	11.1
NADPH–H_2O_2-oxidoreductase	5.0 ± 0.3	38 ± 5	7.6
NADH–*t*-butylhydroperoxide–oxidoreductase	10.0 ± 2.1	140 ± 17	14.0
NADPH–*t*-butylhydroperoxide–oxidoreductase	2.2 ± 0.4	23 ± 4	10.4
SOD (NBT–Xanthine–Xanthine-Oxidase)	770 ± 270	2300 ± 340	3.0

(430 mU mg^{-1} of protein). The induction obtained in the absence of the repressor exceeded previous results for this enzyme activity under microoxic conditions by a factor of 6. Under the same conditions, using NADPH as an electron donor, ROS and oxygen detoxification activity were decreased by a factor of 3–5 when compared with NADH in the same reaction pathways (Table 3).

The detoxification of the highly reactive superoxide radical was less influenced in the absence of PerR. Using the standard SOD assay conditions, which produced superoxide (O_2^-) via xanthine and xanthine oxidase, we detected activity either by the O_2^--mediated reduction of cytochrome *c* (data not shown) or nitro blue tetrazolium chloride (NBT, Table 3). SOD activity which depends on cytochrome *c* reduction has previously been identified in many clostridia. However, low induction under microoxic growth conditions was observed for *C. acetobutylicum* (1.3-fold; Kawasaki *et al.*, 2005). Furthermore, desulfoferrodoxin, a recently characterized superoxide reductase (Riebe *et al.*, 2007), might interfere in a cytochrome *c*-dependent assay, as these enzymes accept reduced cytochrome *c* as an electron donor (Jenney *et al.*, 1999). When cytochrome *c* was substituted for NBT in the assay, we observed an approximately threefold increase in SOD activity in the *perR*-deleted strain (Table 3). Following native gelelectrophoresis, we demonstrated induced NBT-dependent SOD activity in *C. acetobutylicum* Δ*perR* as a visible non-coloured band (Fig. 6). The genome of *C. acetobutylicum* comprises two genes encoding putative SODs. The genes *sodB* and *sodC*, encoding Fe/Mn- and Cu/Zn-type enzymes, respectively, were previously identified, and the transcription of *sodC* was found to be induced by oxygen (Kawasaki *et al.*, 2007). Furthermore, slight SOD activity has been demonstrated for rubrerythrin (Lehmann *et al.*, 1996) and desulfoferredoxin (Riebe *et al.*, 2007).

PerR is involved in negative regulation of oxidative stress genes

As a first approach to give evidence for the role of PerR as a regulatory protein in *C. acetobutylicum* and to identify putative members of the clostridial PerR regulon, we performed two-dimensional gel electrophoresis (2D-PAGE). We expected the expression of oxidative stress proteins would be severely altered in the mutant, even in the absence of stress conditions. Cells of *C. acetobutylicum* and the *perR*-deleted strain were each grown in CGM to the mid-exponential growth phase and were used as a source of protein for 2D-PAGE.

When harvested and disrupted in the presence of oxygen, the cells of the *C. acetobutylicum* Δ*perR* and their extract adopted a deep-red colour with absorption maxima at 377 and 492 nm (Fig. S5), which has previously been reported for purified rubrerythrin-like proteins upon oxidation of their iron sites (LeGall *et al.*, 1988). The

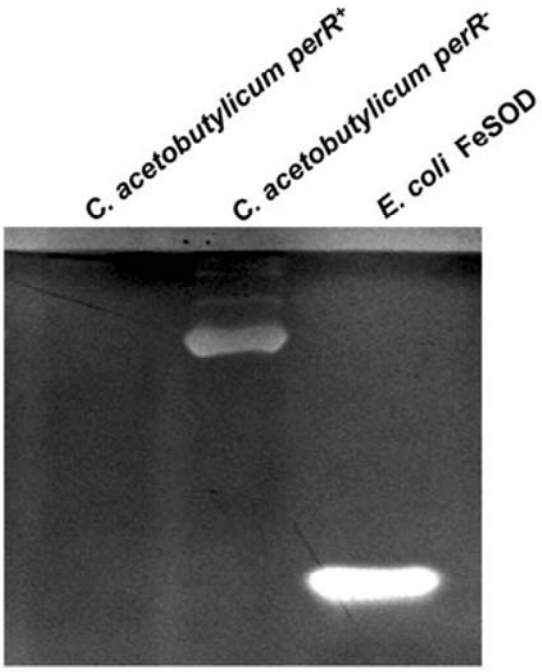

Fig. 6. Superoxide dismutase activity of *C. acetobutylicum*. A non-denaturing polyacrylamide gelelectrophoresis of 100 µg of total cellular protein of wild-type *C. acetobutylicum* (*perR*$^+$) and the deletion mutant (*perR*$^-$) was followed by a SOD activity stain. The iron-dependent enzyme of *E. coli* (*E. coli* Fe-SOD, 2.5 µg) is shown as a control.

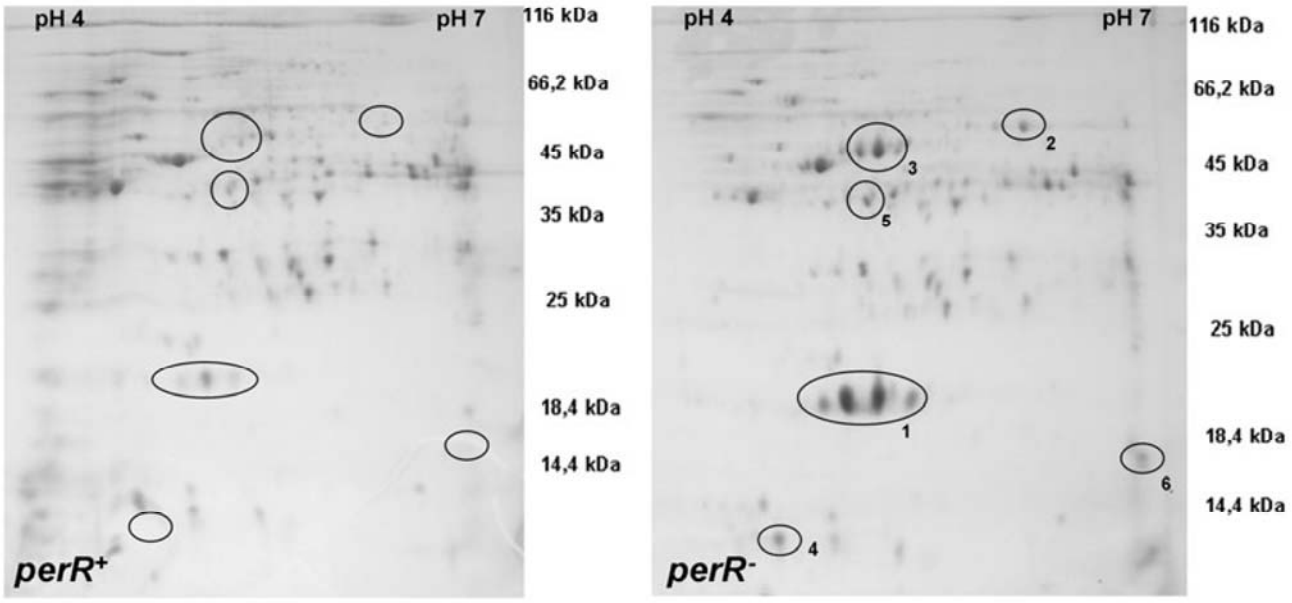

Fig. 7. Protein expression during anaerobiosis of *C. acetobutylicum* (*perR*$^+$) and the deletion mutant (*perR*$^-$) as revealed by two-dimensional gelelectrophoresis. The numbers indicate proteins which are upregulated in the expression profile of the *perR* deletion mutant. Proteins were identified using a mastergel of *C. acetobutylicum* (Fiedler, 2006). 1 and 2, Hsp21; 3, flavoprotein, *cac1027*; 4, flavodoxin, *cac2542*; 5 and 6, not identified.

inference of increased expression of rubrerythrin in *C. acetobutylicum* Δ*perR* was strongly supported by the comparison of the two expression profiles (Fig. 7). In the absence of PerR, the reverse rubrerythrin Hsp21 was the most abundant protein in the cell, and only few other proteins were upregulated. The *perR* mutant revealed four additional proteins which were highly upregulated in the absence of PerR, including the product of *cac1027*, a flavoprotein of unknown function. These two proteins were previously reported to be upregulated in *C. acetobutylicum* in the presence of oxidative stress (Kawasaki *et al.*, 2004; May *et al.*, 2004). Furthermore, PerR-dependent upregulation was determined for the protein product of *cac2452* encoding a flavodoxin which was previously identified as part of a gene cluster which was upregulated in the presence of oxygen (Kawasaki *et al.*, 2005).

In agreement with these data, even in non-stressed *C. acetobutylicum* Δ*perR*, the transcription levels of several members of the putative PerR regulon were raised. All of these were previously identified to be either induced by H_2O_2 or O_2 (Kawasaki *et al.*, 2004; 2005; May *et al.*, 2004). Using probes against *rbr3A* (encoding the Hsp21; May *et al.*, 2004), *dfx* (the superoxide-reducing desulfoferrodoxin; Riebe *et al.*, 2007) and *bcp* (a putative alkylhydroperoxidase; Kawasaki *et al.*, 2005), we observed a substantial difference in the mRNA levels of their respective operons in Northern hybridization experiments (Fig. 8). At the same time, transcription of the normal *rbr* genes, *rbr1* and *rbr2*, was not significantly altered in the mutant, pointing out that reverse and normal rubrerythrins underlie independent regulatory mechanisms in the same organism.

Discussion

In recent years, many efforts have been directed to outline what actually consigns anaerobic organisms to a life in the absence of oxygen and hence, restricts them to anaerobic habitats. Originally, it has been the general opinion that obligate anaerobiosis is tightly linked to the absence of SOD or catalase activities (McCord *et al.*, 1971), while lately, it has been suggested that a fermentative metabolism, employing enzymes extremely sensitive to oxidative inactivation, may strongly interfere with life in the presence of atmospheric oxygen levels (Imlay, 2003; 2006).

In this study, deletion of a *perR*-homologous gene in *C. acetobutylicum*, an organism generally considered as strictly anaerobic, resulted in a phenotype of increased peroxide resistance, but also of almost complete tolerance of an aerobic environment. PerR was identified as a regulatory protein which rather represses oxidative stress genes than being involved in iron uptake, as the level of most metals remained essentially unaltered upon its absence.

As expected from earlier results with aerotolerant bacteria, e.g. *B. subtilis* (Bsat *et al.*, 1998), *S. aureus* (Horsburgh *et al.* 2001) or *S. pyogenes* (King *et al.* 2000), the *perR* deletion mutant of *C. acetobutylicum* was able to tolerate approximately sevenfold higher levels of H_2O_2.

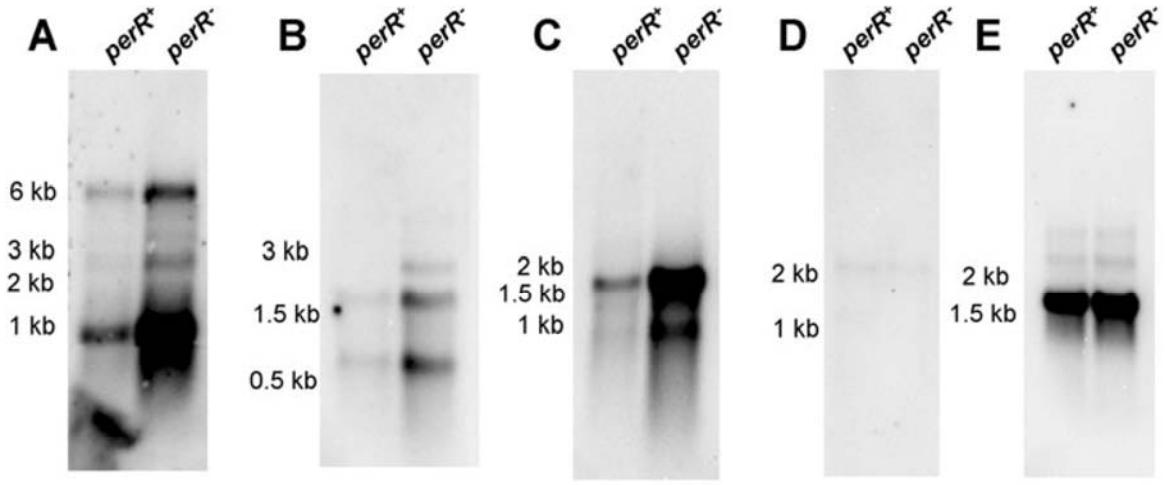

Fig. 8. Expression of oxidative stress genes in *C. acetobutylicum*. For Northern hybridization with RNA from *C. acetobutylicum perR*$^+$ and *perR*$^-$, each lane was loaded with 5 μg of total RNA and hybridized with gene-specific probes against genes encoding (A) desulfoferrodoxin, a superoxide reductase, (B) a bacterioferritin comigratory protein, a putative alkylhydroperoxide reductase, (C) the reverse rubrerythrin Hsp21, (D) the normal rubrerythrin Rbr2 and (E) the 16S rRNA as a control for equal loading. Transcript sizes are indicated in kilobases on the left.

As no catalase activity has been determined so far and no gene encoding this efficient H_2O_2 scavenger is annotated in the genome (Nölling *et al.*, 2001), it is rather unlikely that such a protein contributes to peroxide survival of *C. acetobutylicum*. Moreover, catalase is an efficient scavenger only at high concentrations of H_2O_2, occasionally experienced by aerobes (Seaver and Imlay, 2001). For anaerobes, reducing equivalents, such as NAD(P)H, are plentiful and favour the use of peroxidase which not only avoids the co-production of additional O_2, but also drives H_2O_2 to much lower levels (Imlay, 2002). Typical anaerobic peroxidases in anaerobic bacteria are the wide-spread rubrerythrins, non-heme iron proteins which were shown to deliver electrons from NADH via rubredoxin to H_2O_2 (Lumppio *et al.*, 2001; Weinberg *et al.*, 2004). In agreement with the high peroxidase activity, we observed a drastic induction of the heat shock protein Hsp21, a reverse rubrerythrin, whose expression is raised in the presence of oxidative and other stressors (May *et al.*, 2004, Hillmann *et al.*, 2006). Furthermore, we determined an increase in the detoxification of alkylhydroperoxides, which was essentially an NADH-dependent reduction. A possible candidate enzyme was identified as a bacterioferritin co-migratory protein (*bcp*, *cac0327*), transcriptionally upregulated in *C. acetobutylicum* Δ*perR*. This protein has previously been characterized as a member of a thioredoxin-dependent alkylhydroperoxidase family (Jeong *et al.*, 2000). In the presence of air, the formation of superoxide is an inevitable event often caused by a flavin-dependent, univalent electron transfer to oxygen. In *C. acetobutylicum*, superoxide can be reduced by desulferrodoxin, a recently characterized superoxide reductase (Riebe *et al.*, 2007) whose expression is increased in the presence of oxygen (Kawasaki *et al.*, 2005) and dependent on PerR. Furthermore, dismutase activity as determined in the absence of PerR could provide additional protection from high levels of superoxide.

The aerotolerance of the Δ*perR* strain of *C. acetobutylicum* is a very interesting and surprising attribute of this mutant. Anaerobic microbes do not grow in the presence of atmospheric oxygen levels, but often vary in their tolerance of microoxic or aerobic conditions. For example, *C. acetobutylicum* was observed to grow microaerobically, but only when the rate of O_2 reduction exceeded the rate of O_2 dissolving (Kawasaki *et al.*, 2004). Because oxygen itself can damage central enzymes of the anaerobic metabolism, e.g. the pyruvate-ferredoxin-oxidoreductase (Meinecke *et al.*, 1989), metabolically active cells are especially affected and aerobic culture conditions quickly resulted in a rapid decrease in viable cells (Fig. 3). From this point, it did not seem self-evident that increased resistance to peroxide would also promote aerotolerance.

In contrast to microoxia, our experimental set-up was designed that the flux of oxygen into the culture exceeded the maximum rate of its consumption. An exponentially growing culture of wild-type *C. acetobutylicum* was not able to fully clear air-saturated medium from dissolved oxygen (Fig. 4). As soon as the influx of oxygen was discontinued, wild-type cells took up oxygen at maximum rate of 0.5 fmol h^{-1} $cell^{-1}$ (Fig. 5) and required more than 80 min to lower the concentration of dissolved O_2 below 50 μmol l^{-1}. In same experiment, cells of the *perR*-deleted strain rapidly took up dissolved oxygen at a maximum rate of 16 fmol O_2 h^{-1} $cell^{-1}$, thereby re-establishing anaerobiosis in less than 15 min.

At high cell densities of *C. acetobutylicum* Δ*perR*, a deregulated expression of oxygen reductases leads to a massive oxygen consumption, sufficient for lowering the level of dissolved oxygen in the medium, despite constant shaking in air. Nevertheless, as a result of the rapid diffusion of oxygen over membranes, the intracellular concentration is essentially the same as outside the cell (Ligeza *et al.*, 1998) and should prevent an anaerobic cellular interior. A different scenario might apply when cells were cultured on a solid medium. Colonies of *C. acetobutylicum* Δ*perR* further increased in diameter when conditions were shifted from anaerobic to aerobic, and hence were able to maintain growth in ambient air, but only after anaerobic pre-incubation. Earlier studies on structure and morphology of colonies of *C. acetobutylicum* revealed that they are covered by a proteineacous sheath (Jones *et al.*, 1980) providing additional protection against the deleterious effects of oxygen and possibly creating a microoxic environment sufficient for continued growth of *perR*-deleted cells. Although elevated levels of peroxidases and superoxide-scavenging enzymes additionally attenuate oxidative damage, it seems obvious that another important factor must contribute to the enhanced air tolerance of *C. acetobutylicum* Δ*perR*. Earlier studies in *E. coli* demonstrated that dipyridyl largely precluded iron from the Fenton-based generation of hydroxyl radicals (•OH), the most deleterious of all ROS (Imlay and Linn, 1988; Imlay, 2003). In the presence of this intracellular iron chelator, wild-type *C. acetobutylicum* was not only nearly as protected from peroxide killing as the *perR*-deleted strain, but also revealed extended aerotolerance, giving evidence that iron is crucial determinant for aerobic survival. As a result of the drastically increased expression of the Hsp21 in *C. acetobutylicum* Δ*perR*, oxygen-exposed mutant cells and their extracts adopted the deep-red colour, characteristic of purified rubrerythrin-like proteins upon oxidation of their iron sites (LeGall *et al.*, 1988; Fig. S5). Although the level of cellular iron remained unaffected in the absence of PerR, its disposition in the cell was apparently changed. In *C. acetobutylicum* Δ*perR*, a significant amount of iron is stored in the iron sites of the domains of rubrerythrins, and hence might partially compensate for the absence of dipyridyl.

Our data provide evidence that multiple factors contribute to air tolerance in *C. acetobutylicum*, all triggered by the derepression of a single regulon. Of the rather few proteins which are targets of PerR in *C. acetobutylicum*, the Hsp21, a reverse rubrerythrin originally described as a heat shock protein, plays a central role in the oxidative stress defence. Rubrerythrins are characterized by a unique combination of a rubredoxin-like $FeCys_4$ centre and a four-helix bundle incorporating a non-sulphur, oxo-bridged diiron site, similar to that of ferritin. The order of these two domains may vary and proteins which have an N-terminal $FeCys_4$ site and a C-terminal diiron site were referred to as 'reverse rubrerythrins'. The genome of *C. acetobutylicum* comprises four genes encoding rubrerythrin-like proteins (May *et al.*, 2004). Of these, only the twin gene (*rbr3A/B*)-encoded reverse rubrerythrins were massively synthesized in *C. acetobutylicum* Δ*perR*, while the more distantly related normal rubrerythrins (Rbr1 and Rbr2) are of low expression in the mutant and the wild type. The genomes of all completely sequenced clostridia reveal multiple copies of genes encoding rubrerythrins of the reverse and the normal type (May *et al.*, 2004). So far, normal rubrerythrins which have been characterized, were shown to function as a SOD (Lehmann *et al.*, 1996) in *C. perfringens* or as NAD(P)H-dependent peroxidases in *D. vulgaris* (Lumppio *et al.*, 2001) or *P. furiosus* (Weinberg *et al.*, 2004). In agreement with our results with a *perR*-deleted strain of *C. acetobutylicum*, expression of normal rubrerythrins in the closely related *C. perfringens* was found to be essentially uninfluenced by oxidative stress (Briolat and Reysset, 2002). A recent study on the isolated, recombinant *C. acetobutylicum* Hsp21 revealed that the $FeCys_4$ site of the protein is rapidly oxidized by H_2O_2 and reduced in the presence of *C. acetobutylicum* cell-free extract. It was concluded that this protein functions as a peroxide reductase (Kawasaki *et al.*, 2007). However, the recombinant protein in that study was missing a functional diiron site. Interestingly, diiron four-helix-bundle proteins like rubrerythrins were earlier suggested to act as ancient dioxygen reductases (Gomes *et al.*, 2001). In agreement with the increased consumption of oxygen following the derepressed production of reverse rubrerythrin, we have evidence that this protein plays a crucial role in the reduction of H_2O_2 and also in the consumption of molecular O_2 (O. Riebe, R.J. Fischer, D.M. Kurtz, Jr., H. Bahl, unpublished).

It seems likely that the sequestration of iron and reduction of lethal damages allow a time-limited survival while at the same time active detoxification occurs. In the presence of high levels of reducing equivalents, this detoxification system favours the reduction of oxygen as the feasible scavenging mechanism. However, full clearing of oxygen via reduction requires a massive production of the respective reductases. A constitutive oxygen defence as a result of a loss of PerR would come with high energetic expenses and slow growth under anaerobic conditions. Using the oxidation sensor PerR as an oxygen-dependent switch allows a temporary response to the immediate problem of oxygen exposure. In the presence of oxygen, this switch triggers aerobic survival and, at the same time, the active re-establishment of anaerobiosis, although at the price that growth is attenuated during the period of oxygen consumption. Thus, in an environment which is predominantly anaerobic, repression of these enzymes appears reasonable, and this growth disadvantage might

explain why no spontaneous mutant has evolved. This study reveals that strict anaerobic microbes are subject to an enduring evolutionary selection for the ability to tolerate oxygen using negative regulation as a control mechanism of their defence.

Experimental procedures

Standard procedures

Agar plates and liquid media were prepared anaerobically by using an atmosphere of 90% N_2 and 10% H_2 in an anaerobic chamber (MACS-MG-500, Meintrup DWS, Germany) and according to Breznak and Costilow (1994). Restriction endonucleases and DNA ligase were obtained from NEB, Pwo polymerase from Peqlab, and were either used according to manufacturer's description or as described by Sambrook and Russell (1989). Chemicals and other enzymes, if not indicated otherwise, were purchased either from Sigma Aldrich or Applichem and were at least of analytical grade.

Bacterial strains and growth conditions

All three strains used in this study were derivatives of *C. acetobutylicum ATCC824*. *C. acetobutylicum MGCcac15* which is deleted of the restriction endonuclease *Cac*824I (Soucaille *et al.*, 2006) was used as the parental strain and as experimental reference. The gene deletion in *C. acetobutylicum MGCcac15* (*perR*$^+$), generating *C. acetobutylicum* Δ*perR* (*perR*$^-$), was achieved using homologous recombination as described (Soucaille *et al.*, 2006). The replicative plasmid pREP::*upp* was used as shuttle vector carrying two replicative origins (*ori* for Gram-negative and *repL* for Gram-positive bacteria) and two antibiotic-resistance genes (*mlsR* and *catP*). The *mlsR* gene was flanked by two fragments homologous to the upstream and downstream regions of *perR*. Correct integration at the target gene was verified by polymerase chain reaction analysis (Fig. S2). For complementation, a BamH1 fragment of the gene region of *perR* was ligated in the plasmid pRep::*upp* (Soucaille *et al.*, 2006). The resulting plasmid pRep::*upp-perR* was transformed in *C. acetobutylicum* Δ*perR*, generating strain *C. acetobutylicum* Δ*perR-perR*$^+$. Cells of all strains were maintained as spore suspensions in a modified MS mineral medium (Monot *et al.*, 1982) and stored at (–20°C. The medium was composed of glucose (60 g l^{-1}), KH_2HPO_4 and K_2HPO_4 (each 0.55 g l^{-1}), $MgSO_4 \times 7H_2O$ (0.2 g l^{-1}), $FeSO_4 \times 7H_2O$ (0.011 g l^{-1}), acetic acid (0.23%). After the pH was adjusted to 6.6 with NH_4OH, *p*-aminobenzoic acid (8 mg l^{-1}), biotin (0.08 mg l^{-1}) and MES × H_2O (21.3 g l^{-1}) were added. CGM (Roos *et al.*, 1985; modified) was used as growth medium for all experiments and included yeast extract (BD Biosciences, 5 g l^{-1}), KH_2HPO_4 and K_2HPO_4 (0.75 g l^{-1} each), $FeSO_4 \times 7H_2O$ and $MnSO_4 \times H_2O$ (10 mg l^{-1} each), $MgSO_4 \times 7H_2O$ (0.4 g l^{-1}), NaCl (1 g l^{-1}), asparagin and $(NH4)SO_4$ (2 g l^{-1} each). For isolation of total RNA, protein and all stress experiments cells of the mid-exponential growth phase (OD 0.6–0.7) were used and diluted in CGM where indicated.

Stress experiments and aerobic culture conditions

To analyse the impact of peroxide stress and air, culture samples of 0.5 ml were transferred to an anaerobic chamber and were challenged with either indicated concentrations of H_2O_2 or the equivalent volume of H_2O for 30 min. For the determination of aerotolerance in aerobic liquid culture, 10 ml of cells from each strain was transferred to aerobic conditions by shaking in ambient air at 200 r.p.m. and 37°C in 100 ml Erlenmeyer flasks. When indicated, the cells were exposed to 1 mM or 0.1 mM of the iron chelator 2,2′-dipyridyl (Sigma-Aldrich, Germany) 5 min prior exposure to H_2O_2 or air respectively. Samples were taken before, and at indicated time points after stress was applied.

To determine the number of survivors, the cfu at each time point were determined. Aliquots of appropriate dilutions were plated on Reinforced Clostridial Agar pH 5.8 (RCA, Oxoid, UK) and incubated under anaerobic conditions for 36–48 h at 37°C. The cfu ml^{-1} for each sample were calculated and normalized to the number obtained for the non-stressed control (100%). The values displayed represent the mean value of three independent experiments.

Oxygen measurements

Clostridium acetobutylicum MGCcac15 and the mutant strain *C. acetobutylicum* Δ*perR* were grown anaerobically to mid-exponential phase at OD_{600} of 0.6, before the cultures were aerated with compressed air for 5 min at 37°C. A 3.5 ml cuvette with the optical oxygen sensor attached was immediately filled with the aerated culture aliquots. The oxygen concentration in the cuvette was followed over time by using a Fibox3 – single-channel fibre-optic oxygen metre (Presens GmbH, Germany) as described in detail by Warkentin *et al.* (2007). The cell concentration of each sample was determined in a Thoma counting chamber.

Isolation of cellular components, hybridization and 2D gelelectrophoresis

Isolation of total RNA with a modified hot-phenol procedure, separation in formaldehyde denaturing gel, transfer to a nylon membrane, hybridization with DNA probes and detection were essentially as described previously by Hillmann *et al.* (2006) and Fischer *et al.* (2006). For generation of probes against the mRNA of *dfx*, *bcp*, *rbr3A/B*, *rbr2* and the *16S* rRNA, the following gene-specific oligonucleotides were used: *dfx*, 5′-ttacgtatccaaaaattccg-3′ and 5′-gtttacgcttattgtaacct-3′; *bcp*, 5′-tttatggagataagagaagg-3′ and 5′-ttcaacatgtccattaacc-3′; *rbr3A/B*, 5′-gaaaggatccatgaaaaaatttaaatgtgttgt-3′ and 5′-ttatctgcagtttgaaatatctgtttaataaac-3; *rbr2*, 5′-atacgagctcagcaactacagagg-3′ and 5′-agtgggtaccatgcaatgaca-3′; *16S*, 5′-gtgccagccgccgcgg-3′ and 5′-ccgtcaattcatttaagttt-3′. The preparation of intracellular protein of *C. acetobutylicum* and 2D gelelectrophoresis were carried out following a recently developed standard operating procedure (Schwarz *et al.*, 2007). Spots were re-allocated and identified using a master gel of *C. acetobutylicum ATCC824* (Fiedler, 2006) and Delta 2D software (Decodon, Germany).

Enzyme assays

All following steps in the preparation of protein were performed under anaerobic conditions in an anaerobic chamber. Cells of the *C. acetobutylicum MGCcac15* and of *C. acetobutylicum* Δ*perR* were harvested by centrifugation and disrupted by sonication (Ultraschall Desintegrator Sonopuls HD60, Medizin- und Labortechnik, Germany). Undisrupted cells and cell debris were removed by centrifugation at 15000 *g* for 30 min. Obtained cell extracts were used as enzyme solution in spectrophotometric assays. NADH- and NADPH-dependent oxidase activities were assayed and NADPH oxidases were measured in 1 ml of air-saturated 50 mM MOPS buffer (pH 7.0) including 0.1 mM EDTA and 0.1 mM NAD(P)H at 35°C. The reaction was started by the addition of enzyme solution. The decrease in absorbance at 340 nm, following the addition of protein, was monitored over time with a spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Germany). One unit of activity was defined as the amount of enzyme that catalyses the oxidation of 1 μmol of NAD(P)H per minute. NAD(P)H-dependent peroxide and alkylhydroperoxide reductases were measured under anaerobic conditions in anaerobic 50 mM MOPS buffer (pH 7.0) including 0.1 mM EDTA, 0.1 mM NAD(P)H and variable amounts of anaerobic enzyme at 35°C. The reaction was started by the addition of either 1 mM H_2O_2 or 1 mM tert-butylhydroperoxide and monitored as the decrease in absorbance at 340 nm as described above. One unit of activity was defined as the amount of enzyme that catalyses the oxidation of 1 μmol of NADH or NADPH per minute and was calculated using $\varepsilon_{340} = 6.2\ cm^2\ \mu mol^{-1}$.

Superoxide dismutase activity assays and staining

Superoxide dismutase activity was monitored either spectrophotometrically or in activity stains using standard assays as reported by McCord *et al.* (1971) or Jenney *et al.* (1999). For reaction conditions in photometric assays, 200 μM xanthine and 3.5 μg ml^{-1} xanthine-oxidase were added to 50 mM potassium-phosphate buffer (pH 7.8) containing either 20 μM cytochrome *c* or 200 μM NBT. The increasing absorbance was monitored either at a wavelength of 550 nm (reduced cytochrome *c*) or 560 nm (di-formazan). As SOD inhibits the reduction because of scavenging of O_2^-, one unit of activity was defined as the amount of enzyme which causes a 50% reduction in the increase in absorbance.

For activity stains according to Beauchamp and Fridovich (1971), native polyacrylamide gels with 100 μg of protein from both strains and Fe-SOD from *E. coli* were kept at 4°C and first soaked in a solution of 0.0025% (w/v) NBT in the darkness for 20 min and subsequently incubated for 15 min in 50 mM potassium-phosphate buffer (pH 7.8) containing 0.25% (v/v) TEMED and 0.01% (w/v) riboflavin. When exposed to a light source of 40 W for 2–7 min, protein with SOD activity appeared as a colourless band against a dark-purple background.

Determination of cellular metal content

To quantify selected cellular metals, 10 mg l^{-1} $ZnSO_4 \times 7H_2O$ was added to CGM, and cells grown for 12 h to an OD of 5.3 (wild type) or 4 (Δ*perR*) were harvested by centrifugation, washed twice with 50 mM TrisHCl (pH 8.0) containing 1 mM Na_2EDTA, and washed once with H_2O. After resuspension in 5 ml of H_2O, aliquots of the cells were used for determination of dry weight and for ICP analysis (IRIS Intrepid II XSP, Thermo electron, USA). For ICP measurements, cells were incubated with 5 ml of 37% HCl and heated to 80°C until dried. Following an incubation at 600°C for 5 h, ashes of cells were solubilized in 10 ml of 37% HCl, and the volume was adjusted to 25 ml with H_2O. Measurements were carried out three times for three independent samples and normalized to a control that contained only 10 ml of 37% HCl and 15 ml of H_2O.

BLAST searches, amino acid sequence alignments and phylogenetic analysis

To identify *B. subtilis* Fur-, PerR- and Zur-homologous proteins in annotated microbial genome sequences, BLAST searches (Altschul *et al.*, 1997) were performed. Genomes of completely sequenced clostridia were selected and searched using the complete amino acid sequences from the *B. subtilis* proteins and the TBLASTN program with a Blosum 62 matrix; gap penalties were set at 11 (Existence) and 1 (Extension) (NCBI, http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were carried out with the full amino acid sequences of identified PerR-homologous proteins by using CLUSTALW (Thompson *et al.*, 1994) with a Blosum 62 matrix with a gap opening penalty set at 10 and gap extension penalty set at 0.05. Based on the multiple sequence alignment, a phylogenetic tree was calculated using Clone Manager (Sci Ed central, USA). From the results of the multiple alignments, an un-rooted cladogram was built using the TreeView software (Page, 1996).

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Supplementary material

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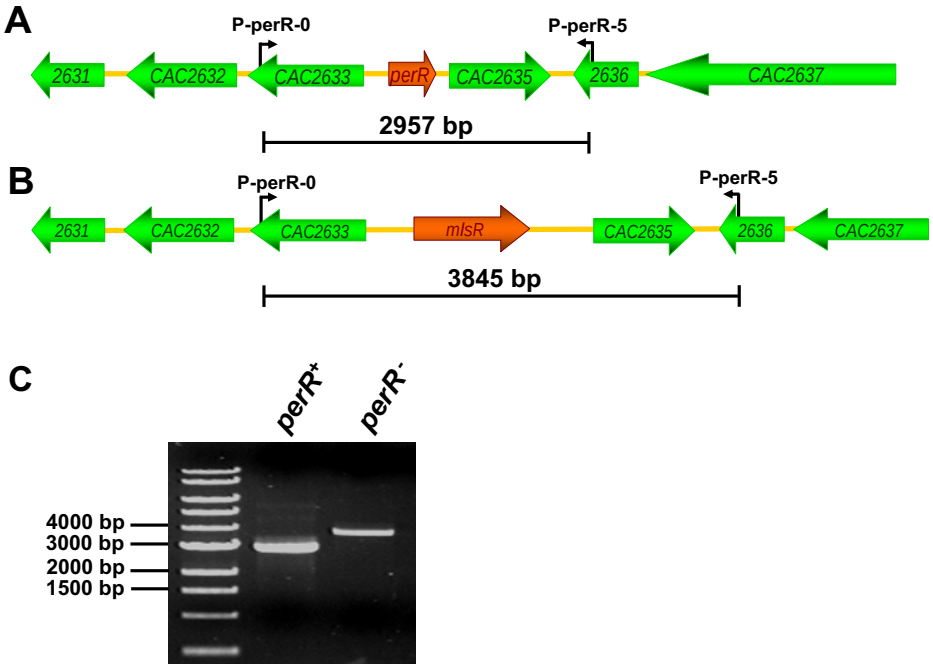
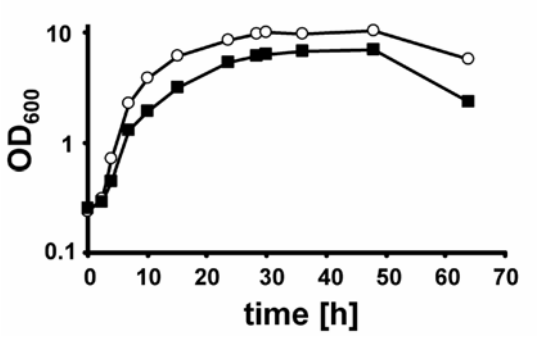
Supplementary Fig. S1. The three Fur-family proteins encoded in the genome of *C. acetobutylicum*. Amino acid sequence alignment reveal the homology of related, previously characterized proteins from *B. subtilis* BsuFur, ferric uptake regulator, acc. No. P54574; BsuPerR, peroxide regulator, acc. No. P71086, BsuZur, zinc uptake regulator, acc. No. 54479. Identical amino acids are in red boxes. Red arrows indicate amino acids putatively involved in metal binding or peroxide sensing of BsuPerR (Lee and Helmann, 2006).

```
BsuFur     1  MENR----IDRIKKQLHSSSYKLTPQREATVRVLLENEEDHLSAEDVYLLVKEKSPEIGLATVYRTLELLTELKVVDKINFG
cac1682    1  MAKLSPLEIEKLKNNLKDKGYKLTPQRRAVLDIIIDNEGQHLTVEELYDLVKLECPEIGLATVYRTVQLLDELGVISKLDLN

BsuPerR    1  ---MAAHELKEALETLKETGVRITPQRHAILEYLVN-SMAHPTADDIYKALEGKFPNMSVATVYNNLRVFRESGLVKELTYG
cac2634    1  --------MNDISTIFKEKKLKLTPQRIAVYKYLKS-THEHPSAETIYKAIQSDYPTMSLATVYKALKTLAEVHLIQELNVG

BsuZur     1  ------MNVQEALNLLKGNGYKYTNKREDMLQLFAD-SDRYLTAKNVLSALNDDYPGLSFDTIYRNLSLYEELGILETTELS
cac0951    1  ---------MDLKTYLKSHNIKITKCRLKILKILNN-ANDALTVETIYKICNDNGKFADLSTVYRSLDLFEEKNVVSKVDLG

BsuFur    79  -DGVSRYDLRKEGAAHFHHHLVCMEFGAVDEIEGDLLEDVEEIIERDWKFKIKDHRLTFHGICHRCNGKETE---- 149
cac1682   83  -DGLSRYELVHENEPHQHHHLICTACGKVTEVQGDLLDELEDCIENKYHFLIKNHSVKFYGICSECRKKL------ 151

BsuPerR   79  -DASSRFDFVTSD----HYHAICENCGKIVDFHYPGLDEVEQLAAHVTGFKVSHHRLEIYGVCQECSKKENH---- 145
cac2634   74  -EGNFRYDANSSS----HPHIQCLSCGKVDDIMGITFDNLNKDVSSHTDYDVISNKLYFYGICKDCKDKA------ 138

BsuZur    76  GEKLFRFKCSFTH---HHHHFICLACGKTKEIESCPMDKLCDDLD---GYQVSGHKFEIYGTCPDCTAENQENTTA 145
cac0951   73  -NGKYSYKIKGDT---HKHVLKCNMCNKEIEIE-CPMDQVEEIIKNKTGFVLIDHELKMKALCKDCMDKSNNEKNS 143
```

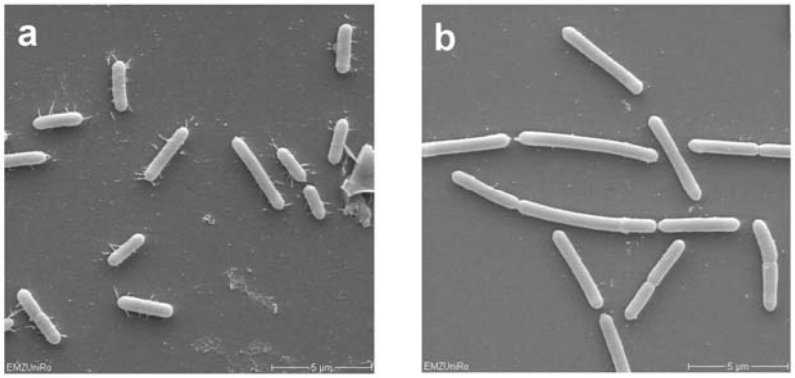
Supplementary Fig. S2. Deletion of a *perR*-homologous gene in *C. acetobutylicum* Oligonucelotides P-perR-0 (5'-aaaaaactgcagtgtaatacattgtagtagatgctccacc-3') and P-perR-5 (5'-aaaaaactgcaggaaactctgaagtttaatgatagtgacg-3') were designed to analyze the DNA-region around *perR* in *C. acetobutylicum perR*$^+$ (**A**) or *perR*$^-$ (**B**). **C**, PCR products of the two strains using P-perR-0 and P-perR-5 as primers.



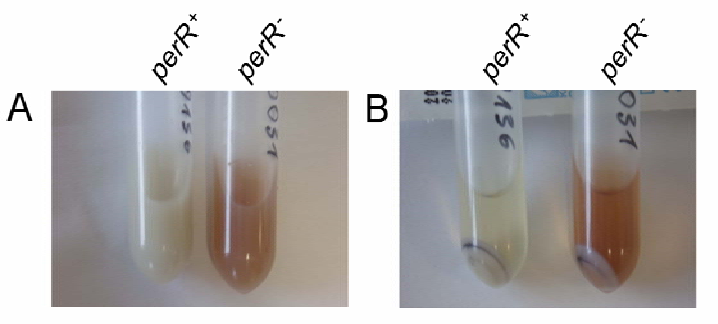
Supplementary Fig. S3. Anaerobic Growth of *C. acetobutylicum perR*$^{+}$ (open circles) and *perR*$^{-}$ (closed squares) in MS mineral medium as described in Experimental procedures. Growth rates (μ) for *C. acetobutylicum perR*$^{+}$ and *C. acetobutylicum perR*$^{-}$ were calculated as 0.47 ± 0.05 h^{-1} and 0.36 ± 0.05 h^{-1}, respectively.



Supplementary Fig. S4. Scanning electron micrograph of cells of *C. acetobutylicum perR*$^{+}$ (**a**) and *perR*$^{-}$ (**b**) after anaerobic growth to an OD_{600} of 0.7 in CGM.



Supplementary Fig. S5. Cells (**A**) and cell-free extracts (**B**) of *C. acetobutylicum* after 5 min incubation in aerobic buffer. Cells were concentrated by a factor of 10. The red colour indicates the increased expression of rubrerythrin-like proteins following the deletion of *perR.*



3.3 Reductive dioxygen scavenging by flavo-diiron proteins of *Clostridium acetobutylicum*

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Die aerotolerante *perR*-Deletionsmutante von *C. acetobutylicum* reduzierte gelösten O_2 mit einer ca. 14fach höheren Rate als der Wildtyp (HILLMANN *et al.*, 2008). Der Artikel beschreibt die O_2- und NO-Reduktaseaktivität der beiden bisher unbekannten Flavoproteine FprA1 und FprA2, die sowohl unter O_2 als auch in der Abwesenheit ihres Repressors PerR eine stark induzierte Expression zeigten (KAWASAKI *et al.*, 2005; HILLMANN *et al.*, 2008). Die Flavoproteine FprA1 (*CAC1027*) und FprA2 (*CAC2449*) wiesen Sequenzähnlichkeiten zu bereits bekannten Rubredoxin-abhängigen O_2- und NO-Reduktasen aus Eubakterien und Archaeen auf (Table 1). Zunächst wurden beide Gene heterolog in *E. coli* überexprimiert und aufgereinigt (Fig. 1). Das Molekulargewicht des nativen FprA1 entsprach einem Homodimer, während für FprA2 eine tetramere Struktur ermittelt wurde (Fig. 2a). Aufgrund ihres Cofaktors, eines Flavinmononukleotids (FMN) pro Monomer, sowie der Existenz einer konservierten Eisenbindedomäne zählen beide zur Familie der „flavodiironproteins“ (Flavo-2Fe-Proteine, Fig. 2b und 3). Beide Proteine waren in der Lage Elektronen von reduziertem Rubredoxin auf O_2 oder NO zu übertragen. Reduziertes Rubredoxin wurde kontinuierlich durch die ebenfalls heterologe NADH-abhängige Rubredoxin-Oxidoreduktase hergestellt. Dieser Elektronentransport war deshalb auch als NADH-Oxidation photometrisch messbar (Fig. 4). Die stöchiometrische Reduktion des O_2 wurde zusätzlich direkt mit einer O_2-Optode gemessen (WARKENTIN *et al.*, 2007). Diese war im Vergleich zur NADH Oxidation um die Hälfte reduziert, wodurch H_2O als Reaktionsprodukt ermittelt wurde (Fig. 5). Die spezifische Aktivität des FprA2 war annähernd zweifach höher als die des FprA1. Diese *in vitro* Versuche bewiesen, dass beide Polypeptide die Fähigkeit besitzen O_2 und NO zu reduzieren und Bestandteil des anaeroben Detoxifikationssystems bei oxidativem und nitrosativem Stress sind (Fig. 6).

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Reductive dioxygen scavenging by flavo-diiron proteins of *Clostridium acetobutylicum*

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ABSTRACT

Two flavo-diiron proteins (FDPs), FprA1 and FprA2, are up-regulated when the strictly anaerobic solvent producer, *Clostridium acetobutylicum*, is exposed to dioxygen. These two FDPs were purified following heterologous overexpression in *Escherichia coli* as N-terminal *Strep*-tag fusion proteins. The recombinant FprA1 and FprA2 were found to be homodimeric and homotetrameric, respectively, and both FDPs functioned as terminal components of NADH oxidases (NADH:O_2 oxidoreductases) when using *C. acetobutylicum* NADH:rubredoxin oxidoreductase (NROR) and rubredoxin (Rd) as electron transport intermediaries. Both FDPs catalyzed the four-electron reduction of molecular oxygen to water with similar specific activities. The results are consistent with these FDPs functioning as efficient scavengers of intracellular dioxygen under aerobic or microoxic growth conditions.

1. Introduction

Obligately anaerobic microorganisms by definition cannot use molecular oxygen as terminal electron acceptor for growth. Obligate anaerobes are, nevertheless, far from defenceless against periodic influxes of O_2 and its even more reactive reduced forms, superoxide and hydrogen peroxide. While aerobic organisms use catalase or superoxide dismutases to lower high levels of hydrogen peroxide (H_2O_2) or superoxide (O_2^-), anaerobes use their relatively high levels of reducing equivalents for reduction rather than dismutation of these molecules, thereby avoiding the regeneration of O_2 [1,2]. Enzymes catalyzing reductive superoxide and peroxide scavenging, namely, superoxide reductase (SOR) and rubrerythrin (Rbr), respectively, have been characterized from a number of anaerobic bacteria and archaea [3–7].

Many anaerobic bacteria also share the ability to reductively scavenge molecular oxygen directly, thereby rapidly re-establishing anaerobiosis upon intermittent exposure to a dioxygenic environment and protecting crucial O_2-sensitive metabolic enzymes [1,2]. These scavenging enzymes typically function as NADH:O_2 oxidoreductases (NADH oxidases) and can be distinguished by their production of either hydrogen peroxide or water from dioxygen reduction [8–10]. The strictly anaerobic, gram-positive solvent producer, *Clostridium acetobutylicum*, can withstand air exposure upon activation of its oxidative stress response, resulting in rapid cellular O_2 consumption and high NADH oxidase activity in crude extracts [11]. However a corresponding gene or protein has not been conclusively identified in this organism up to now. Upon exposure of *C. acetobutylicum* to aerobic or microoxic (5% O_2) atmospheres, or during activation of its PerR (peroxide response regulator) regulon only a very few proteins were detectably up-regulated, most prominently Hsp21, which was subsequently identified as reverse rubrerythrin (revRbr) [12,13]. Although revRbr may participate in O_2 scavenging, we have shown that its preferred substrate is H_2O_2 [7].

Two other candidates for O_2-scavenging enzymes in *C. acetobutylicum* are the putative flavo-diiron proteins (FDPs), FprA1 and FprA2. Expressions of their respective genes, *cac1027* and *cac2449*, are inducible by exposure to a microoxic atmosphere [13,14] and both of these FDPs were shown to be highly abundant in an aerotolerant *perR* deletion strain of *C. acetobutylicum* [11]. *Cac2449* was identified as part of an O_2-responsive gene cluster [14], which also encodes an NADH:rubredoxin oxidoreductase (NROR) (*cac2448*) [7], and a SOR (*cac2450*) [6]. FDPs are widespread in anaerobic bacteria and archaea [15–17], and even occur in a few primitive eukaryotes [18]. The characteristic features of FDPs are an N-terminal non-heme diiron domain and a C-terminal

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flavodoxin-like domain [15–18]. The amino acid sequence motifs for both of these domains, as well as all the iron-ligating residues, are conserved in *C. acetobutylicum* FprA1 and FprA2 (see Fig. 1 and Table S1). FDPs from several sources have been shown in vitro to function as the terminal components of NADH oxidases [15–18]. This activity requires the participation of electron transfer intermediary proteins, such as NROR and rubredoxin (Rd), between NADH and FDP. Genes encoding the small electron transfer protein, Rd or Rd-like protein, are in fact, co-transcribed with those of FDP homologs in other bacteria [17,19,20]. The NADH oxidase activity has led to the proposal that at least some FDPs function as O_2 scavengers in vivo. To our knowledge only putative FDPs from clostridia have been shown to be up-regulated upon O_2 exposure [14,19], yet no clostridial FDPs have been characterized. We, therefore, investigated whether the *cac1027* and *cac2449* gene products are in fact FDPs, whether they can function as the terminal components of NADH oxidases and whether this activity is consistent with an O_2 scavenging function in vivo.

2. Materials and methods

2.1. Reagents, enzymes and standard procedures

Reagents and buffers used for cloning. purification and enzyme assays were at least of analytical grade. Restriction endonucleases and DNA ligase, obtained from NEB and Pwo polymerase from Peqlab were either used according to the manufacturer's description or as described elsewhere [21]. Unless indicated otherwise, chemicals were purchased either from Sigma–Aldrich or Applichem and were at least of analytical grade. Amino acid sequence alignments were carried out using the Clustal W software [22]. Iron and protein analyses were carried out as previously described [17].

2.2. Cloning, expression, and purification of proteins

The genes encoding FprA1 (*cac1027*) and FprA2 (*cac2449*) were amplified from chromosomal DNA of *C. acetobutylicum* by PCR using the oligonucleotides *P1_CAC1027*-BamHI 5′-AAAAGGATCC-AGTGCTGAAAAGCTTTGTG-3′, *P2_CAC1027*-PstI 5′-AAAACTGCAG-TAATAAACCTATAAAATCATC-3′ for *fprA1*, and *P1_CAC2449*-BamH1 5′-AAAAGGATCCCCAGCTATAAAAATTAAAG-3′ and *P2_cac2449-PstI* 5′-AAAACTGCAGTATACTTTTGGCAAAGTC-3′ for *fprA2* as primers, introducing BamHI and PstI restriction sites. The resulting fragments were ligated in the pASK-IBA7+ expression vector (IBA-Go) which allows expression of strep tag fusion proteins from an anhydrotetracyclin (AHT) inducible promoter [23]. The resulting plasmids pI7*fprA1* and pI7*fprA2* were transformed in competent *Escherichia coli* DH5α cells. The recombinant strains were grown in 500 ml of Luria–Bertani (LB) medium with 100 μg ml^{-1} of ampicillin at 30 °C and 120 rpm on a rotary shaker. At an OD_{600} of 0.3–0.5, 0.2 μg ml^{-1} of AHT were added to induce protein expression. Cells were harvested by centrifugation following growth for 10–12 h. Strains expressing recombinant Rd and NROR from pI3rbo_{cac} and pT$nror_{cac}$, respectively, were grown as described previously [6,7]. Cell pellets were either stored for up to 7 days at −20 °C or immediately used as a source of cellular protein. All further purification steps of the heterologous proteins used in this study (Rd_{cac}, $NROR_{cac}$, FprA1 and FprA2) were carried out as described previously [7]. The protein content in the elution fractions was determined using the Bradford assay [24]. Reduced forms of the proteins were obtained by the addition of a stoichiometric amount of sodium dithionite. Purification of the proteins was analyzed using 12.5% Coomassie-stained SDS–PAGE gels.

A more thermostable NROR from *Thermotoga maritima* ($NROR_{tma}$) was used for determination of Michaelis–Menten parameters. (The *T. maritima* NROR gene (TM0754) is adjacent to that encoding the *T. maritima* FDP homolog (TM0755)). *E. coli* expression strains for N-terminal His-tagged $NROR_{tma}$ (TM0754) and Rd (TM0659) from *T. maritima* were obtained from the Joint Center for Structural Genomics (http://www.jcsg.org/) and are described on the Harvard Institute of Proteomics Plasmid ID website (http://plasmid.med.harvard.edu/PLASMID/GetCloneDetail.do?cloneid=85043&species=). The expression strains were grown in LB at 37 °C to an OD_{600} of 0.6–1.0, and protein expression was induced with 0.02% arabinose. The cultures were incubated for an additional 3–4 h at 37 °C, and the cells were then harvested and lysed by sonication. The recombinant $NROR_{tma}$ and Rd_{tma} were purified from the cell lysate using Talon® columns (Clontech) with an elution buffer consisting of 50 mM MOPS, 250 mM NaCl, 250 mM imidazole, pH 7.3.

2.3. Determination of molecular weights

Molecular weights of the native proteins were determined by analytical gel filtration and fast pressure liquid chromatography (FPLC, Pharmacia Biotech) using a Superose 12 10/300 column (GE Healthcare) and 50 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, and

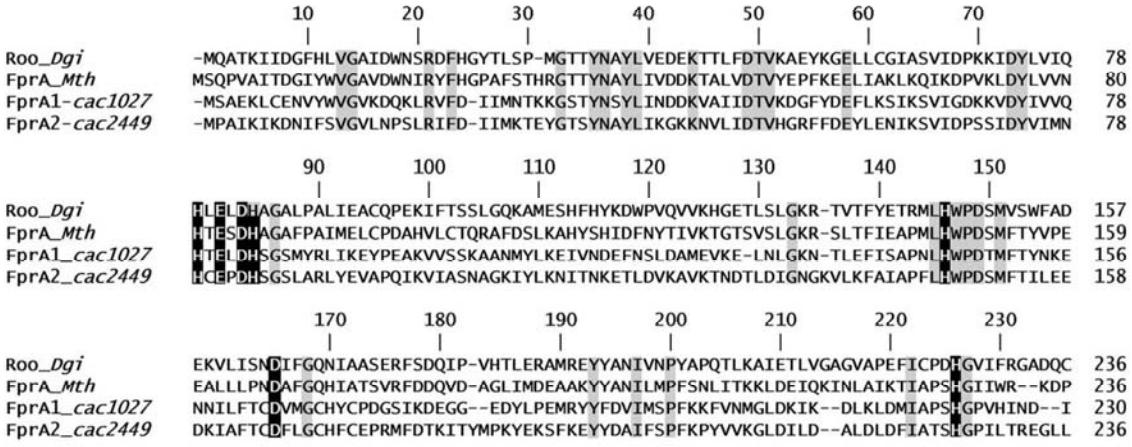

Fig. 1. Amino acid sequence alignment of the N-terminal diiron domains of FDPs from *Moorella thermoacetica* (*Mta*), *Desulfovibrio gigas* (*Dgi*), (a.k.a. rubredoxin:oxygen oxidoreductase, Roo), and *Clostridium acetobutylicum* FprA1 and FprA2. Identities among the four proteins are shaded in gray. The six conserved residues which furnish iron ligands are marked by black boxes.

150 mM NaCl as the mobile phase. The column was calibrated with proteins of known sizes: aldolase, 158 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; and chymotrypsinogen, 25 kDa.

2.4. NADH oxidase and NADH:nitric oxide oxidoreductase activities

NADH-dependent oxidase activities were measured spectrophotometrically essentially as previously described [6,7] at 25 °C in 0.4 ml of air-saturated 50 mM *N*-morpholinopropanesulfonic acid (MOPS) buffer + 0.1 mM EDTA, pH 7.0. The standard assay contained in the order of addition 100 μM of NADH, 2 μM of Rd_{cac}, 0.1 μM of $NROR_{cac}$, and 1 μM (monomer concentrations) of either FprA1 or FprA2. Protein concentrations were determined by the Bradford method [24] and the molecular weights. The rate of decrease in absorbance at 340 nm resulting from NADH oxidation was monitored using a Ultrospec 3000 spectrophotometer (Pharmacia Biotech). One unit of activity was defined as the amount of FDP in milligrams which catalyzed the oxidation of 1 μmol of NADH using $\varepsilon_{340} = 6.2\ cm^{-1}\ mM^{-1}$.

Michaelis–Menten parameters for NADH oxidase and NADH:nitric oxide oxidoreductase (NOR) activities were determined at room temperature (~23 °C) in 50 mM MOPS, pH 7.3, by measurements of initial rates of NADH consumption (ΔA_{340nm}/min) as a function of dioxygen or nitric oxide concentrations. Measurements were carried out in 1-ml septum-capped cuvettes in an N_2-filled Vacuum Atmospheres Company glove box using an Ocean Optics USB2000 spectrophotometer. The assay mixtures contained 200 μM NADH, saturating concentrations of $NROR_{tma}$ and Rd_{tma} (4 μM and 10 μM, respectively), and FprA2 monomer concentrations of 100 nM (for NADH oxidase) or 40 nM (for NOR). Protein concentrations were determined using $\varepsilon_{450nm} = 12\,000\ M^{-1}\ cm^{-1}$ for both $NROR_{tma}$ and FprA2 monomer and $\varepsilon_{492nm} = 8870\ M^{-1}\ cm^{-1}$ for Rd_{tma} [25]. Reactions were initiated by syringe injections of appropriate volumes of either air- or nitric oxide-saturated MOPS buffer. The nitric oxide gas (99.5%, Praxair) used to prepare the saturated solutions was first passed through a concentrated KOH solution to remove higher oxides of nitrogen [18].

2.5. O_2 reduction by FprA1 and FprA2 in the presence of Rd, NROR and NADH

The reduction of O_2 was monitored at room temperature (~23 °C) as the decrease in the concentration of dissolved O_2 vs time under the NADH oxidase assay conditions described above (using *C. acetobutylicum* proteins) except in a volume of 4 mL using the Fibox3 – single-channel fibre-optic oxygen metre (Presens). The operating mode of this device is described elsewhere [26].

3. Results

3.1. Purification and characterization of the C. acetobutylicum FDPs

The *C. acetobutylicum* genes, *cac1027*, encoding FprA1, and *cac2449*, encoding FprA2, were heterologously expressed as N-terminal *Strep*-tag® fusion proteins in *E. coli* DH5α and purified in one step by *Strep*-Tactin® affinity column chromatography [23]. This protocol yielded highly pure FprA1 and FprA2 (Fig. 2) at 2-8 mg l^{-1} of culture. Gel filtration of the purified recombinant proteins (see Fig. S1) revealed distinct oligomeric structures: a homodimer for FprA1 (99 ± 5 kDa, approximately twice the calculated monomer mass of 48.4 kDa), and homotetramer for FprA2 (191 ± 11 kDa, approximately four times the calculated monomer mass of 49.7 kDa). Storage of FprA2 (48 h, 4 °C) showed additional peaks at 104 ± 6 kDa and 49 ± 1 kDa corresponding to the formation of dimers and monomers, respectively (data not shown). The purified proteins were bright yellow due to the UV–visible absorption features at 378 nm and 450 nm of the oxidized flavin cofactors, as shown for FprA1 in Fig. 3. TLC analysis identified FMN as the flavin cofactor in both FprA1 and FprA2 (data not shown), as is invariably found in all characterized FDPs together with a diiron site [15–18,25,27]. Analyses of FprA2 showed iron/FMN/protein monomer ratios of 2.3/1.0/1.0. Thus, each subunit of these FDPs contains one FMN and one diiron site, i.e., one active site per FprA1/2 monomer.

3.2. FprA1 and FprA2 function as terminal components of an NADH oxidase

FprA1 and FprA2 (at 1 μM monomer concentrations), by themselves, did not show detectable NADH oxidase activity (data not shown). This result is not surprising, since FDPs from other organisms invariably require oxidoreductase intermediaries for NADH oxidase activity [15–18]. The *C. acetobutylicum* NROR [10] is part of a gene cluster that encodes FprA2. We, therefore, tested whether the purified NROR and Rd could together function as NADH:FprA1 and FprA2 oxidoreductases in NADH oxidase assays. $NROR_{cac}$ alone

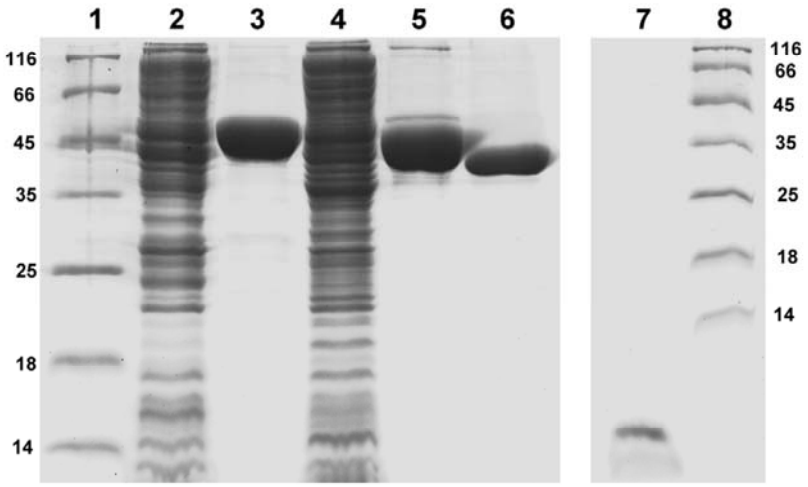

Fig. 2. SDS–PAGE analysis of heterologous overexpression and purification of *C. acetobutylicum Strep*-tagged® FprA1, FprA2, $NROR_{cac}$. Lanes 1 and 8, molecular weight marker proteins; lanes 2 and 4, crude extracts of induced *Escherichia coli* DH5α-pl7*fprA1* and -pl7*fprA2*, respectively; lanes 3, 5, 6, and 7, *Strep*-tactin® column elution fractions containing FprA1, FprA2, $NROR_{cac}$ and Rd_{cac}, respectively.

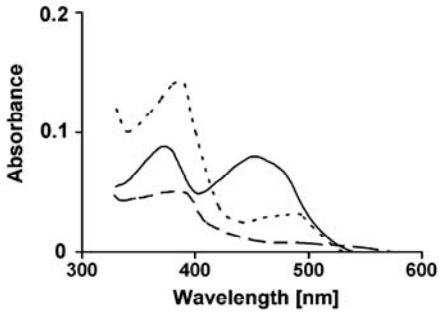

Fig. 3. Visible absorption spectra of oxidized (as-isolated) (—), partially dithionite reduced (---) and fully dithionite reduced (— —) *C. acetobutylicum* FprA1.

had very low NADH oxidase activity (<0.2 U mg^{-1}), but this activity drastically increased (>30-fold) when Rd_{cac}, and either FprA1 or FprA2 were added (Fig. 4). This activity was strongly dependent on the presence of all three proteins. In an assay system composed of 0.1 μM $NROR_{cac}$, 2 μM Rd_{cac}, and 1 μM FprA1 or FprA2 (monomer concentration) NADH was consumed at a maximum rate of 127 ± 9 μmol min^{-1} per μmol of FprA1 (6.6 U mg^{-1}) and 211 ± 14 μmol min^{-1} per μmol of FprA2 (11.0 U mg^{-1}). NADPH consumption was not observed when it was substituted for NADH in this assay system. The *C. acetobutylicum* NROR has previously been shown to efficiently catalyze reduction of Rd by NADH but not by NADPH [6,7]. Thus NROR and Rd function as intermediate components in the electron transfer chain: NADH → NROR → Rd → FprA1/2, in which Rd and not the NROR serves as the proximal electron donor to the FDPs.

To verify that FprA1 or FprA2 were indeed acting as the terminal component of NADH oxidase, we directly measured O_2 consumption at ~23 °C using an oxygen electrode in solutions corresponding to the assay mixtures of Fig. 4. Thus, $NROR_{cac}$, Rd_{cac}, and either FprA1 or FprA2 were sequentially added to O_2-saturated buffer (~260 μmol l^{-1}). Upon addition of FprA1/2 O_2 was consumed at a maximum rate of 51 ± 6 μmol O_2 min^{-1} per μmol of FprA1 and 92 ± 9 μmol O_2 min^{-1} per μmol of FprA2 (Fig. 5). These FDPs could conceivably catalyze reduction of O_2 to either H_2O or H_2O_2 according to reactions (1) or (2), respectively:

$$2NADH + 2H^+ + O_2 \rightarrow 2NAD^+ + 2H_2O \quad \text{(reaction1)}$$

$$NADH + H^+ + O_2 \rightarrow NAD^+ + H_2O_2 \quad \text{(reaction 2)}$$

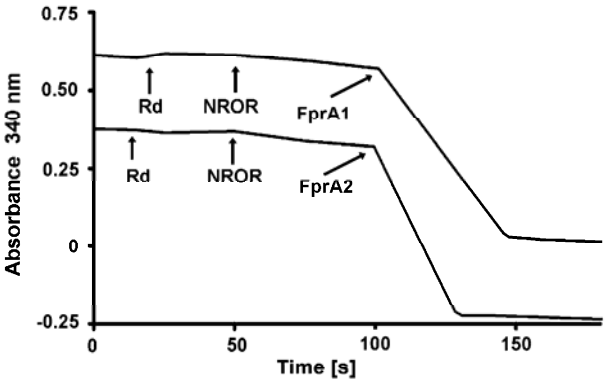

Fig. 4. NADH oxidase activity of $NROR_{cac}$, Rd_{cac} and FprA1 or FprA2. NADH or NADPH consumption was measured as decrease in absorbance at 340 nm. The assay contained 100 μM of NADH, 2 μM of Rd_{cac}, 0.1 μM of $NROR_{cac}$, and 1 μM of either FrpA1 or FprA2. Arrows indicate the time points of protein additions. For clarity both time courses are offset by 0.25 absorbance units.

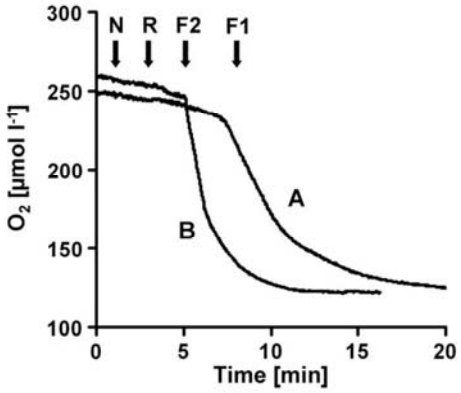

Fig. 5. O_2 consumption by $NROR_{cac}$, Rd_{cac} and FprA1 or FprA2 in the presence of NADH. The decrease of the O_2 concentration was monitored over time using a fibre-optic O_2 metre, as described in Section 2. The assay mixtures contained initially 250 μM NADH in air-saturated 50 mM MOPS + 0.1 mM EDTA, pH 7.0, followed by additions of small volumes of proteins to achieve concentrations of 0.1 μM for $NROR_{cac}$, 2 μM for Rd_{cac}, and 1 μM (monomer basis) for either FprA1 (A) or FprA2 (B). Arrows indicate the time points of protein additions: N, NROR; R, Rd; F1, FprA1; F2, FprA2.

The mole ratios of NADH oxidation/O_2 consumption (127 ± 9 μmol NADH/51 ± 6 μmol O_2 = 2.5 for FprA1, and 211 ± 14 μmol NADH/92 ± 9 μmol O_2 = 2.3 for FprA2). are much closer to the 2:1 NADH:O_2 stoichiometry of reaction (1) than the 1:1 stoichiometry of reaction (2). These results, thus, show that H_2O is the predominant product of O_2 reduction by both FprA1 and FprA2, as has been invariably observed for other FDPs [15–18].

3.3. NADH oxidase vs NADH:nitric oxide oxidoreductase catalytic efficiencies

Because some FDPs function as terminal components of an anaerobic NADH:nitric oxide oxidoreductase (NOR) [17,25,27–29], we compared the catalytic efficiencies of the NADH oxidase and NOR activities of FprA2 under conditions of saturating NROR and Rd at ~23 °C. For these determinations the higher-yield and more robust *T. maritima* NROR ($NROR_{tma}$) homolog was used for the multiple repetitive initial rate measurements at various O_2 or NO concentrations. The data shown in Fig. S2 yielded k_{cat}, K_m, and k_{cat}/K_m values of 5 s^{-1}, 16 μM, and 3×10^5 s^{-1} M^{-1}, respectively, for the NADH oxidase and 34 s^{-1}, 40 μM, and 1×10^6 s^{-1} M^{-1} for the NOR activities. These values are similar to those reported for other FDPs [25], and indicate that FprA2's NADH oxidase and NOR catalytic competencies are similar to each other. An improved fit to the NOR activity data (Fig. S2) was obtained using a kinetic model in which the required two nitric oxides per turnover show cooperative binding to the diiron site, as has been observed for other FDPs [17,25]. Such cooperativity is neither expected nor observed for the NADH oxidase activity, which requires only a single O_2 per active site per turnover.

4. Discussion

The induction of various types of oxidative stress responses allows anaerobic microbes to colonize habitats that are subject to periodic influxes of O_2. *C. acetobutylicum* is capable of time-limited survival in microoxic environments [13], and has been shown to rapidly consume molecular O_2 from an aerobic environment upon full activation of its PerR regulon [11]. The results reported here show that the two *C. acetobutylicum* FDPs, FprA1 and FprA2, which are highly expressed in response to O_2 exposure [13,14], are able to serve as the terminal component of a reconstituted NADH oxidase,

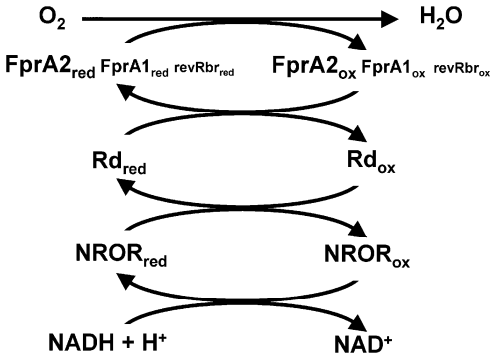

Fig. 6. Model for O_2 scavenging in *C. acetobutylicum*. NROR, NADH:rubredoxin oxidoreductase; Rd, rubredoxin; FprA1, flavo-diiron protein 1; FprA2, flavo-diiron protein 2; revRbr, reverse rubrerythrin; red, reduced; ox, oxidized.

catalyzing the four-electron reduction of molecular oxygen to water. Both FprA1 and FprA2 contain the characteristic FMN and diiron cofactors, the latter of which is the site of O_2 reduction in other FDPs [17]. The electron transfer pathway from NADH to O_2 (Fig. 6) can be reconstituted using the native *C. acetobutylicum* oxidoreductase components. The NADH oxidase specific activities of FprA1/2 determined in this work are at least 100-fold higher than that of the revRbr using the same electron transfer components and similar assay conditions [7]. Therefore, although revRbr is also highly induced upon O_2 exposure and highly overexpressed in the aerotolerant perR deletion strain [11], its predominant function is most likely as a scavenger of its preferred substrate, H_2O_2.

Many (although not all) FDPs have been shown to serve as terminal components of anaerobic NADH:nitric oxide oxidoreductases (NORs), thereby scavenging toxic nitric oxide [17,25,27–29]. The *E. coli* FDP homolog, flavorubredoxin, is in fact induced in response to anaerobic nitric oxide exposure [29]. We show in this work that FprA2 can serve as the terminal component of an NOR with a catalytic efficiency comparable to that of its NADH oxidase activity. Future investigations of the two *C. acetobutylicum* FDPs will include their in vivo roles in scavenging nitric oxide.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.12.004.

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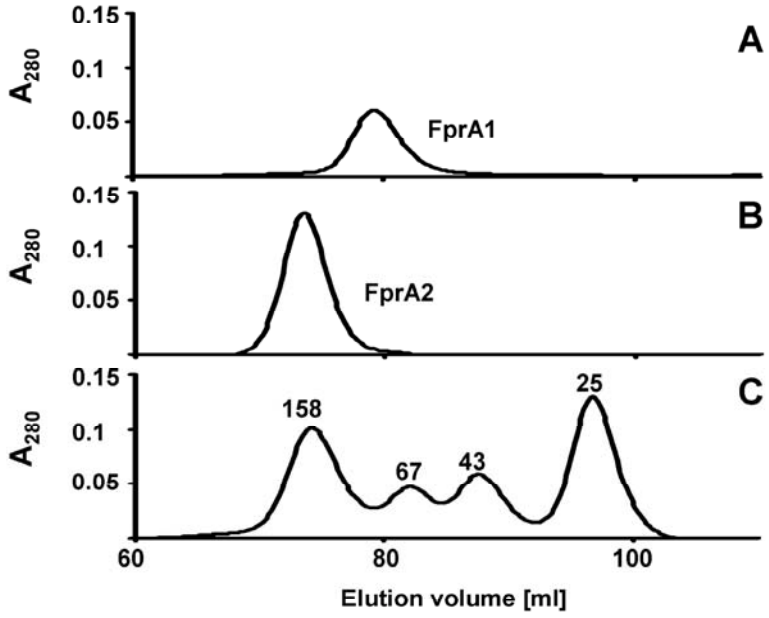
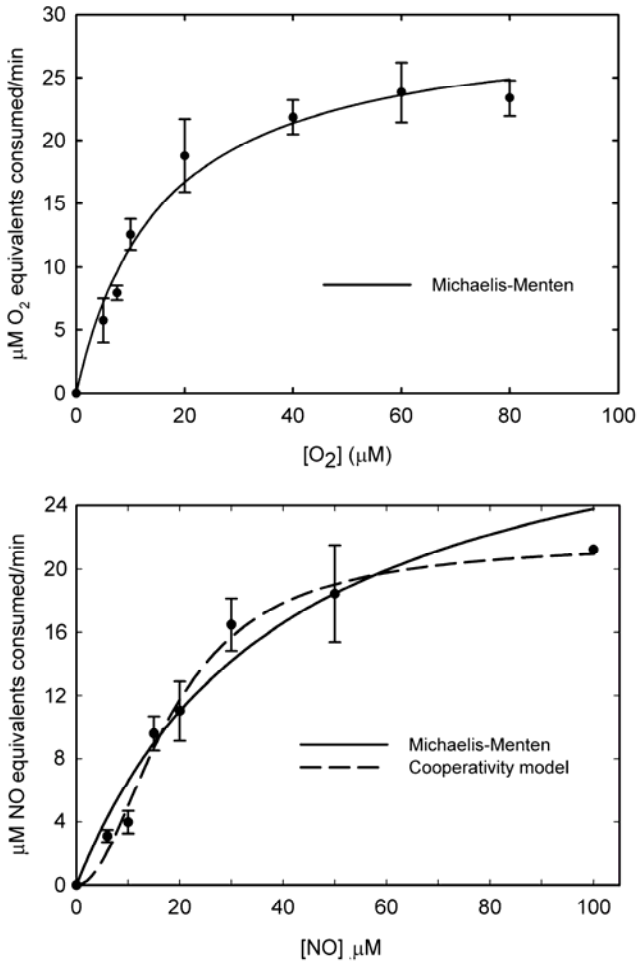
Supplementary Table S1. Homology of FprA1 and FprA2 form *Clostridium acetobutylicum*, CLOAB, to previously characterized FDPs from *Desulfovibrio gigas*, DESGI; *Moorella thermoacetica*, MOOTA; *Methanothermobacter arboriphilus*, 9EURY;*Thermotoga maritima*, THEMA.

	Amino acid residue identity / similarity (%)				
	FprA1 Q97K92_CLOAB	**Roo** Q9F0J6_DESGI	**FprA** Q9FDN7_MOOTA	**$F_{420}H_2$ oxidase** B1A7S3_9EURY	**Roo** Q9WZL4_THEMA
FprA1 Q97K92_CLOAB	**100 / 100**	**30 / 48**	**34 / 54**	**32 / 49**	**26 / 49**
FprA2 Q97GC0_CLOAB	**40 / 61**	**28 / 48**	**30 / 50**	**29 / 46**	**29 / 48**

Supplementary Fig. S1. Analytical gel filtration of Strep-Tactin-purified *C. acetobutylicum* FprA1 (A), FprA2 (B) and proteins with known sizes (C, aldolase, 158 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; and chymotrypsinogen, 25 kDa), as described in Materials and Methods.



Supplementary Fig. S2. Michaelis-Menten plots for NADH oxidase (top panel) and NOR (bottom panel) activities of *C. acetobutylicum* FprA2. Assay conditions are described in Materials and Methods using FprA2 subunit concentrations of 100 nM for NADH oxidase and 40 nM for NOR activities. Each data point represents the average of three determinations with error bars indicating the range. Solid curves are fits of the Michaelis-Menten equation to the data, yielding the parameters listed in the main text. Fits of a cooperativity model for the NOR activity (described elsewhere [26]) yielded the dashed curve with parameters, K_1, K_2, and k_{cat} of 480 µM, 0.7 µM, and 9 s^{-1}, respectively.



3.4 The role of PerR in O_2 affected gene expression of *Clostridium acetobutylicum*

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Manuskript in Vorbereitung

Die Aerotoleranz der *perR*-Deletionsmutante war nicht nur durch hohe Reduktionsaktivität zu erklären. Es war daher notwendig die Rolle des PerR-Regulons in der O_2-Antwort von *C. acetobutylicum* genauer zu definieren. Weiterhin war von Interesse wie PerR in Gegenwart von O_2 die Induktion von Zielgenen vermittelt. Hierzu wurden globale Transkriptionsanalysen durchgeführt. Diese ermöglichten den Vergleich der Genexpression zwischen anaerob und mikroaerob wachsenden Zellen von *C. acetobutylicum*, sowie zwischen dem Wildtyp- und dem *perR*-deletierten Stamm unter anaeroben Bedingungen. In Gegenwart von O_2 reagierte *C. acetobutylicum* mit der stark induzierten Expression von Genen deren Proteinprodukte besonders dem Detoxifikationsystem, dem Energiestoffwechsel, und dem Transport von anorganischen Ionen zugeordnet werden konnten. Insgesamt wurden unter O_2 68 Gene mehr als dreifach stärker exprimiert. Für 15 Gene wurde O_2-abhängig eine dreifach geringere Transkription festgestellt (Fig. 1, Table 2). Nicht alle Gene des O_2-Stimulons waren Teil des PerR-Regulons. Dies zeigte, dass weitere, PerR-unabhängige Regulationsmechanismen unter O_2 existieren. Das PerR-Regulon umfasste alle Komponenten des alternativen Detoxifikationsweges, aber auch einige zentrale Elemente des Energiestoffwechsels (Table 2). Vor Genen mit der höchsten Induktion konnte im Promotorbereich das AT-reiche Palindrom identifiziert werden (Table 3). Aufgrund seiner Konserviertheit und der Bindung von heterologem PerR an die *rbr3A/B* Promotorregion wurde eine PerR-Box postuliert (Fig. 2 und Fig. 3). Eine Komplementation einer *B. subtilis perR*-Defektmutante mit dem *perR* Gen aus *C. acetobutylicum* führte zu massiv erhöhter H_2O_2 Empfindlichkeit und Repression der Katalase bei aerobem Wachstum (Fig. 4 und Fig. 5). Dies verwies auf die funktionelle Homologie zum Protein aus *B. subtilis* und zeigte, dass u. a. intrazelluläres H_2O_2 als Sensor innerhalb des globalen O_2-Regulationssystems von *C. acetobutylicum* (Fig. 6) verwendet wird.

ABSTRACT

In the strict anaerobe *C. acetobutylicum* a PerR homologous protein has recently been identified as a key repressor of a reductive machinery for the scavenging of reactive oxygen species and molecular O_2. In the absence of PerR full derepression of its regulon resulted in increased resistence to oxidative stress and nearly full tolerance of an aerobic environment. In the present study, complementation of a *Bacillus subtilis* PerR mutant confirmed that the homologous protein from *C. acetobutylicum* acts as a functional peroxide sensor *in vivo*. Furthemore, we used a transcriptomic approach to analyze the gene expression in the aerotolerant PerR mutant strain and compared it to the O_2 stimulon of wild type *C. acetobutylicum*. The genes encoding the components of the alternative detoxification system were PerR regulated. Only few other targets of direct PerR regulation were identified, including two highly expressed genes encoding enzymes putatively involved in the central energy metabolism. All of them were highly induced when wild type cells were exposed to sublethal levels of O_2. Under these conditions *C. acetobutylicum* also activated the repair and biogenesis of DNA and Fe-S clusters, as well as the transcription of a gene encoding an unknown CO dehydrogenase like enzyme. Surprisingly few genes were downregulated under O_2 including those involved in butyrate formation. In summary, these results show that the defence against oxidative stress of this strict anaerobe is robust and by far not limited to the removal of O_2 and its reactive derivatives.

INTRODUCTION

Though many bacteria are commonly classified as strict anaerobes, aerotolerance can vary greatly among these organisms. Some anaerobes even use catalase and superoxide dismutase as efficient H_2O_2 and O_2^- scavenging enzymes that were earlier proposed to be exclusively limited to aerobes (47, for reviews see 5 and 32). Even when missing these two, fermentative anaerobes can often use their replete pool of reducing equivalents for the complete reduction of these molecules and O_2 itself. In anaerobes in particular the generation of the two major reactive oxygen species (ROS), H_2O_2 and O_2^- is elevated during oxygen exposure due to their wide use of reduced flavins as cofactors in redox enzymes. Current models for the anaerobic oxygen detoxification via superoxide reductase, peroxidase and NADH oxidases have been proposed for *Pyrococcus furiosus, Desulfovibrio vulgaris*, and most recently for *Clostridium acetobutylicum* (33, 60, 70). These essentially include oxygen reducing flavodiiron proteins (FDPs), superoxide reducing desulfoferrodoxin (Dfx), and rubrerythrins as peroxidases (26, 60). Reduced rubredoxin thereby acts as the intermediate electron carrier,

which pool is continuously reloaded by an NADH dependent rubredoxin dependent oxidoreductase (58, 60). The sporulating Gram positive *C. acetobutylicum* is a classic example of a fermentative strict anaerobe. In the absence of oxygen it ferments sugars to the organic acids acetate and butyrate or shifts to solvent formation with acetone and butanol as major products (for review see 12). Especially the latter one has regained attention lately as an attractive biofuel (13). Thus, metabolic engineering and *in silico* modelling of the clostridial metabolism are ranked highly among recent efforts to increase productivity (42, 56, 62, 65). Furthermore, the sequenced genome and the design of microarrays for global-scale transcriptional analysis in *C. acetobutylicum* have substantially improved the understanding of solvent formation and allowed a detailed view on its unique life cycle (34, 52, 56).

During exposure to sublethal O_2 concentration *C. acetobutylicum* rapidly induces the expression of all components of its detoxification system and consumes dissolved O_2 (25). However, when the rate of influx exceeds the rate of consumption the organism immediately ceases its metabolic activity and resumes only after anaerobiosis is restored (36, 54). Studies on central redox enzymes employed by anaerobes revealed that these were highly susceptible to damage of their iron sulphur sites caused directly by molecular oxygen, e. g. pyruvate-formate lyase (PFL) of *Escherichia coli*, or pyruvate-ferredoxin oxidoreductase (PFOR) from *C. acetobutylicum* (48, 62). *Bacteroides thetaiotaomicron*, a Gram negative anaerobe is even employing efficient repair mechanisms to recycle this enzyme once conditions are shifted back to anaerobic (55).

Altogether, these observations have established the concept that anaerobes are not primarily sensitive to oxygen due to their lack or inefficiency of scavenging enzymes. Instead central metabolic steps in the anaerobic energy conversion are poisoned by molecular oxygen (29,31). So, it does not seem surprising that some anaerobes which are temporarily, but frequently exposed to oxygen found ways to avoid these bottlenecks and direct the carbon flow onto oxygen resistant pathways (55, 68). A similar strategy has been proposed for an oxygen resistant mutant of *C. acetobutylicum* (31). Following deletion of the clostridial *perR* homologue constitutive expression of its regulon resulted in drastically enhanced aerobic survival and permitted time limited growth in an aerobic environment (25). In closely related facultative aerobes like *Bacillus subtilis* the peroxide repressor PerR senses intracellular levels of H_2O_2 by metal catalyzed histidine oxidation and controls genes which proteins are involved in scavenging of hydrogen peroxide, iron storage and DNA protection (22, 40). PerR regulation has also been proposed for anaerobic sulphate reducers and was further supported by coordinate induction of the predicted target genes in response to low oxygen concentrations with *Desulfovibrio vulgaris*. (49, 61). The work presented here is a combined approach of microarray technology and complementational studies to obtain a global view of the organism's oxygen stimulon and refine the regulatory role of PerR in a strict anaerobe.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All strains used in this study are listed in Table 1. *C. acetobutylicum* MGC*cac15* and *C. acetobutylicum ΔperR* were stored as spore suspensions at -20°C and grown in an MS minimal medium as described previously (25). Growth was followed as the optical density at a wavelength of 600 nm. Cells for RNA sampling were anaerobically grown in 1 l serum flasks and at OD_{600} of 0.6-0.7 cultures were either aerated with compressed air with a rate of 0.05 l air min^{-1} or left anaerobic as a control. The concentration of dissolved O_2 was measured using the WTW OXI 196 oxygen meter (WTW, Weilheim, Germany). As a result of continuous uptake of O_2 from the growth medium its concentration stayed essentially below detection (<6.25 µmol l^{-1}) Samples for RNA isolation were taken following 1 h of O_2 exposure or from anaerobic controls at an OD of 0.6-0.8. *E. coli* pT*perR*$_{Cac}$ constitutively expressing the clostridial PerR homologue was grown overnight in 500 ml of LB medium with 100 µg ml^{-1} of Ampicillin at 30°C and 120 rpm on a rotary shaker. *B. subtilis* strains were grown at 30°C and 170 rpm in LB-medium. For cultivation on solid medium, 15 g l^{-1} of Agar were added to the medium. To select for *B. subtilis* mutant strains 10 µg ml^{-1} kanamycin (*fur*$^-$) and 100 µg ml^{-1} streptomycin (*perR*$^-$) were added as antibiotics. Complemented strains carrying the colstridial *perR* gene were additionally selected by the addition of 10 µg ml^{-1} Chloramphenicol and 0.5 % (w/v) of xylose was added to induce expression *perR*$_{Cac}$ expression.

TABLE 1. Bacterial strains used in this study

	Strain	Relevant characteristics[a]	reference
C. acetobutylicum			
	MGC*cac*15	ATCC824, *Δcac824I*	67
	ΔperR	MGC*cac*15, *ΔperR, mlsR*$^+$	25
E. coli			
	pT*perR*$_{Cac}$	DH5α, plasmid pT*perR*$_{Cac}$, *amp*$^+$	this study
B. subtilis			
	HB1000	ZB307A *attSPβ*	7
	HB1000R$_{Cac}$	HB1000 *amyE*::*perR*$_{Cac}$, *cat*$^+$	this study
	HB6543	HB1000 *fur::kan*	6
	HB6543R$_{Cac}$	HB6543 *amyE*::*perR*$_{Cac}$, *cat*$^+$	this study
	HB0509	HB1000 *perR::spc*	6
	HB0509R$_{Cac}$	HB0509 *amyE*::*perR*$_{Cac}$, *cat*$^+$	this study

[a] Abbreviations: *amp*$^+$, ampicillin resistance gene; *cat*$^+$, chloramphenicol resistance gene; *kan*, kanamycin resistance gene; *spc*, spectinomycin resistence gene, *mlsR*$^+$, macrolide, lincosamide, and streptogramin B resistant

Construction of plasmids. For the heterologous overexpression of the clostridial PerR protein, its gene was PCR-amplified from chromosomal DNA of *C. acetobutylicum* using the oligonucleotides P_TSH-*CAC*2634-*Bam*HI with 5'-AAAA*GGATCC*AACGATATATCTACAA-3' and P_TSH-*CAC*2634-*Xma*I with 5'-AAAA*CCCGGG*AGCTTTATCCTTACAG-3' as primers,

introducing *Bam*HI and *Xma*I restriction sites. Following purification and restriction, the PCR fragment was exchanged for the *hydA* gene in the pT*hydA* vector described by Girbal *et al.* (18) and the resulting pT*perR*$_{Cac}$ was transformed in *E. coli* DH5α cells. Recombinant cells were selected by the addition of ampicillin and constitutively expressed PerR from the clostridial thiolase promoter with fused Streptag at the C-terminus.

For the complementation of *B. subtilis* strains the *C. acetobutylicum perR* gene was amplified from chromosomal DNA using P_perR-pX-1 with 5'-AAAA*TCTAGA*CACGTTTTCGAAAGCAAGG-3' and P_perR-pX-2 with 5'-AAAA*GGATCC*TTACAACTAGCAATATTTG-3' introducing *Xba*I and *Bam*HI restriction sites, respectively. The amplified fragments were cloned in the pX vector which integrates in the *amyE* locus of *B. subtilis* and allows xylose inducible expression of proteins (38). The recombinant vector was transformed in *B. subtilis* strains HB1000 (wild type), HB6543 (*fur*$^-$), and HB0509 (*perR*$^-$), using the method described by Cuttingham and Youngman (9). Positive transformants were selected due to their resistance to 10 µg ml^{-1} of Chloramphenicol.

RNA isolation, labelling and microarray analysis. *C. acetobutylicum* cell samples for isolation of total RNA were pelleted by centrifugation, immediately shock-frozen in liquid N_2 and stored at -70°C. RNA was isolated using a modified Hot-Phenol procedure as described by Fischer *et al.* (16). To avoid DNA contamination, isolated RNA was digested with 20 U of RNase-free DNase (Amersham Pharmacia Biotech, Freiburg, Germany) in a total volume of 50 µl and incubated for 30 min at 37°C. The RNA was cleared from DNase by an addition of 15 µl of 2 M sodium acetate pH 5.2, additional treatment with phenol, and precipitation with ethanol. Dried RNA was dissolved in 30 µl of H_2O and the quality of these preparations was controlled by agarose gel electrophoresis. RNA was reverse transcribed to cDNA and labeled with fluorescent Cy-3 and Cy-5 dyes using the direct labeling protocol of the CyScribe first-strand cDNA labelling kit (GE Healthcare, Freiburg, Germany) as described (28). To check the labelling efficiency, the amount of labelled cDNA was quantified using the molar extinction coefficients of Cy-3 and Cy-5 and a nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). DNA microarray slides were spotted with two complete sets of PCR products, each covering 99.8 % of the annotated *C. acetobutylicum* open reading frames. Slides were hybridized with aliquots of the cDNA preparations at 42°C overnight in a Lucidea SlidePro hybridization chamber (GE Healthcare, Munich, Germany). Hybridization and wash steps were essentially as described by Hovey *et al.* (28). Following hybridization fluorescence signal intensities were collected for each spot using a GenePix 4100A scanner. Normalization and evaluation of data was carried out with the GenPix Pro software version 6.0 (Axon Instruments, Union City, USA). RNA was isolated from three independent cultures of *C. acetobutylicum* MGC*cac15* which were either left anaerobic or exposed to air. RNA of the *perR* mutant was derived from three anaerobically growing cultures of *C. acetobutylicum ΔperR*. RNA from *C. acetobutylicum* from anaerobic cultures was compared to RNA derived

from aerated samples of the same strain, and to RNA derived from anaerobic cultures of *C. acetobutylicum ΔperR*. To avoid dye specific effects RNA from each sample was labelled with both dyes and dye-swap experiments were performed. As a single slide gave two hybridization signals for each ORF, a total of 12 sets of transcriptional data were obtained for each gene. Differential expression of an ORF was regarded as significant when the average value of the ratio median from at least 8 datapoints was either ≥ 2 or ≤ 0.5. Full lists of transcribed genes, functional information and regulation under O_2 or in *perR* deleted cells are available as online supplementary material with this article (Table S1 and S2).

Promoter analysis and motif identification. The virtual foot print software linked to the PRODORIC database (http://prodoric.tu-bs.de/vfp; 50) on gene regulation and gene expression in prokaryotes was used to identify DNA regions with high similarity to the proposed regulatory motif IR1. The sequenced genome of *C. acetobutylicum* including the megaplasmid pSOL1 was searched for the palindromic nucleotide sequence 5'-AATNNNTATTANNTAATANNNATT-3', with "N" representing any type of nucleotide and allowing one mismatch. The search pattern was identified in 13 different positions on the chromosome. Two sites were located in the coding region of genes and eleven sites were in the 5' non-coding region of genes within 250 bp distance to the starting codon. Motifs with three mismatches were also identified in the upstream region of two other highly upregulated genes. A sequence logo representing a multilevel consensus sequence for each motif was achieved by alignment of these sequences with the weblogo software accessible through the website: http://www.weblogo.berkeley.edu (8).

Heterologous overexpression and protein purification. PerR from *C. acetobutylicum* was heterologously expressed as a *Strep*-Tag fusion protein in *E. coli* cells carrying the plasmid pT*perR*$_{Cac}$. Cells were harvested by centrifugation, and washed in a 50 mM TrisHCl-buffer (pH 8.0) with 150 mM NaCl and 1 mM EDTA. Pellets were either stored for up to 7 days at -20°C or immediately used as a source of protein. All further purification steps of the heterologous protein used in this study were carried out as described previously (59). The protein content in the elution fractions was determined using the Bradford assay (4).

Electrophoretic mobility shift assay. A DNA fragment of the *rbr3A/B* promoter region, covering the last 235 bp of the *rbr3A* 5' untranslated region was PCR amplified using the oligonucleotides P_3p_*rbr3A* with 5'-AGTGTCTGCAGAAGCAGGGAAAAG-3' and P_5p_*rbr3A* with 5'-AATTTTCTAGATTAATCTCTCTCA-3' as primers. Reactions were carried out with *Pwo* DNA polymerase (Peqlab, Erlangen, Germany) with chromosomal DNA from *C. acetobutylicum* ATCC 824 as a template. PCR products were purified from agarose gels with the Nucleospin Extract II Kit (Macherey-Nagel, Düren, Germany) and 3'-Digoxygenin-labelled using the DIG gel shift kit, 2nd generation (Roche Applied Science, Mannheim, Germany) following the

manufacturer's instructions. Labelled promoter fragments (12.5 nM) were incubated for 20 min at room temperature with 25 to 50 nM of purified $PerR_{Cac}$. Buffer conditions were 20 mM TrisHCl, 5 % Glycerol, 5 ng μl^{-1} salmon sperm DNA, 50 ng μl^{-1} BSA, 50 mM KCl, 100 µM $MnCl_2$. Unlabelled fragments were added as controls for specific binding to DNA. The reactions were applied to 1.5 % (w/v) agarose gels with 0.5 x TBE (44.5 mM Tris-HCl [pH 8.0], 44.5 mM boric acid, 5 mM EDTA) as running buffer. Electrophoreses were run at room temperature for 1-1.5 h at 65 V and the DNA fragments were blotted on nylon membranes (Nytran SuPerCharge Nylon Transfer Membrane, 0.45 µm pore size, Schleicher & Schuell Bioscience, Dassel, Germany). Detection of the DIG-labelled DNA was performed as described previously (46).

Zone of inhibition assay. To determine the sensitivity to H_2O_2 a zone of inhibition assay was performed essentially as described by Bsat *et al.* (6). Fresh LB medium (50 ml) with the appropriate antibiotics was inoculated with *B. subtilis* from overnight cultures and cells were grown at 30°C at 170 rpm on a rotary shaker to the mid-logarithmic growth phase (OD_{600} 0.4-0.5). Aliquots of 1 ml were added to 15 ml of prewarmed (45°C) LB containing 1.5 % (w/v) of Agar without antibiotics. The cell suspension was poured into agarplates and after hardening 5 mm filter paper discs containing 5 µl of 1 M H_2O_2 were placed on the agarplates. Growth and zones of inhibition for each strain were monitored following an overnight incubation at 30°C. Each strain was analyzed in triplicate. Results for each strain were reproducible within a range of ± 15 % of the maximum diameter of the inhibition zone.

Measurement of katalase activity. *B. subtilis* cells were grown to the late exponential growth phase (OD_{600} of 1.0-1.2) in LB medium and harvested by centrifugation. Pellets were stored at -20°C or immediately used for the preparation of crude extracts. Cells were disrupted by sonication at 4°C using the Ultraschall Desintegrator Sonopuls HD60 (Medizin- und Labortechnik, Hamburg, Germany) and undisrupted cells and cell debris were removed by centrifugation at 15000 g for 30 min. The protein concentration in obtained cell extracts was determined with the Bradford assay (4).Catalase activtiy was measured spectrophotometrically at a wavelength of 240 nm. Different amounts of enzyme were added to a 50 mM Potassium Phosphate Buffer (pH 7.0) at 25°C and blanked. The reaction was started by the addition of H_2O_2 up to a final concentration of 25 mM. The decrease in absorbance was followed over time using an Ultrospec 3000 spectrophotometer (Pharmacia Biotech, Germany). One unit of catalase was defined as the amount of enzyme that decomposes 1 µmol of H_2O_2 min^{-1} using ε_{240} = 43.6 mM^{-1} cm^{-1}.

RESULTS

Oxygen leads to a global transcriptional response in *C. acetobutylicum.* To identify O_2 inducible genes, anaerobic cultures of *C. acetobutylicum* were sparged with compressed air at a rate of 0.05 l min^{-1} l^{-1} culture volume during the logarithmic growth phase. As a result of continuous uptake the O_2 concentration remained below detection limits (<0.1 μmol^{-1}) and growth continued albeit at a slightly reduced rate (data not shown). Samples for the analysis of global transcription were taken following one hour of microaerobiosis in during the exponential growth phase. The gene expression of aerated cells was compared to a parallel culture which was left anaerobic. Oxygen exposure drastically effected (> 3fold) the transcription of 83 of the 3848 protein coding genes (3672 on the chromosome and 176 on the megaplasmid pSOL1) resulting in 68 genes which were induced and 15 genes which were repressed. The COG (cluster of orthologous groups) classification of proteins allowed a first functional overview of the products of all genes which were differentially expressed (> 2fold) under O_2 (Tatusov *et al.*, 2000; Fig. 1A). However, a large proportion (40 % of the upregulated genes and 27 % of the downregulated genes) encoded proteins which could not be grouped or were of unknown function (X). Most dramatic changes occurred with genes which proteins belonging to the COG clusters C (energy production and conversion; 7.6 % of upregulated and 15 % of downregulated genes), E (amino acid transport and metabolism; 8.4 %, and 2 %, respectively), G (carbohydrate metabolism and transport; 4,7 %, and 10 %, respectively), H (Coenzyme transport and metabolism; 2.2 %, and 12 % respectively), and P, (inorganic ion transport, 7 %, and 6 %, respectively). Overall, theses changes in the global transcription suggested that *C. acetobutylicum* actively reacts to oxygen in the environment by concentrating its energy resources on the protection of essential metabolic pathways, maintenance of a reducing interior, and detoxification of reactive oxygen species.

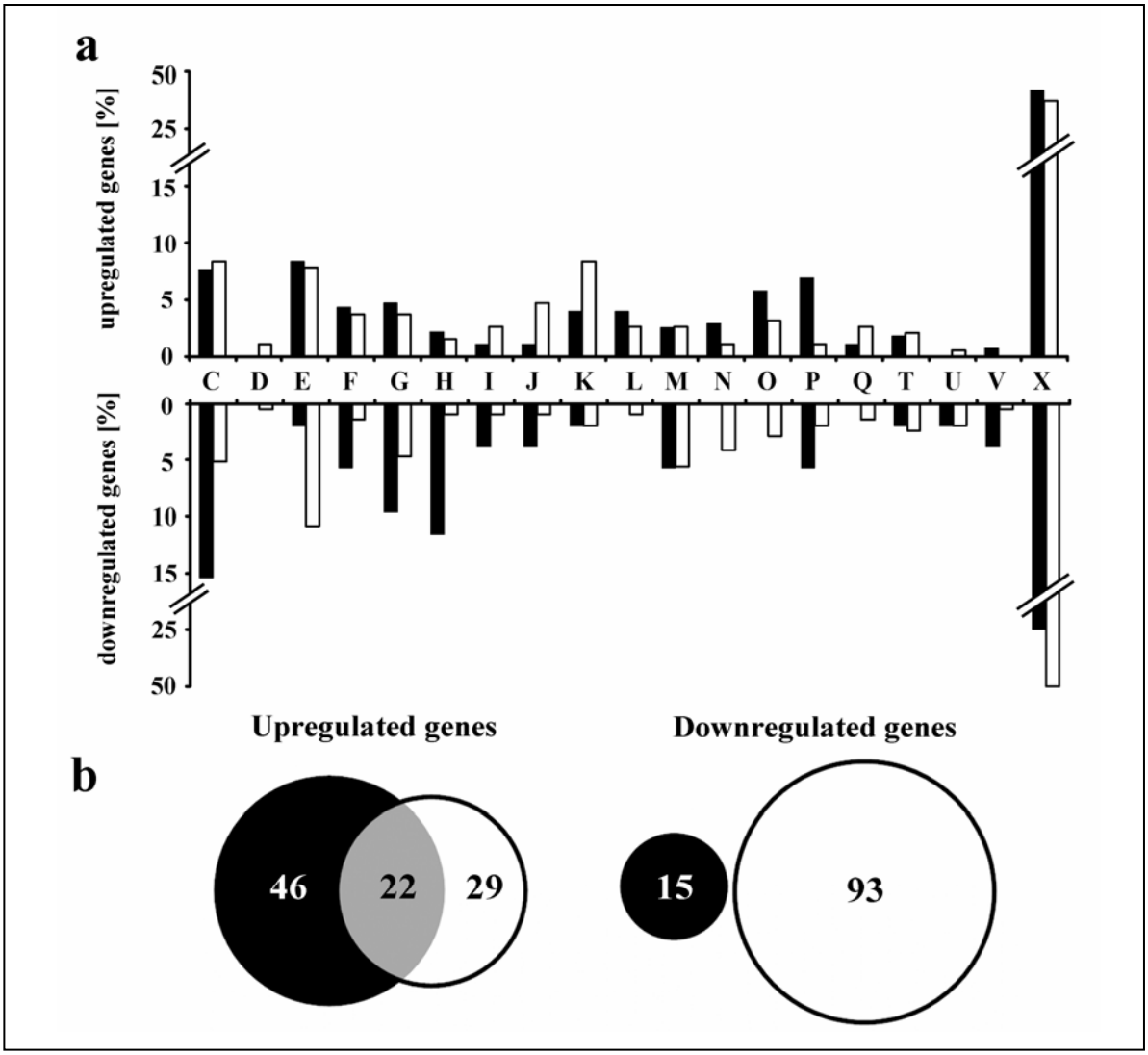

FIG.1. Global transcriptional changes in wild type *C. acetobutylicum* under O_2 and in *C. acetobutylicum* lacking PerR.

(a) The proteins products of differentially regulated genes (>2fold) were grouped according to the COG classification scheme. Black and shaded columns indicate O_2 and $PerR^-$ responsive genes, respectively. The COG designations are as follows: C, energy; D, cell cycle control, cell division; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism and transport; I, lipid metabolism; J, translation; K, transcription and regulation; L, replication, recombination and repair; M, cell wall and membrane proteins; N, cell motility; O, protein fate and chaperones; P, inorganic ion metabolism and transport; Q, secondary metabolites; T, signal transduction; U, secretion and intracellular trafficking; V, defence; X, poorly characterized or no cluster.

(b) Ven diagrams illustrating the overlapping (grey) 3fold induction (left) or 3fold repression (right) of members of the oxygen stimulon (black circle) and the PerR regulon (white circle).

Proteins involved in detoxification and redox balance. The group of genes which were highly activated included all those which encoded the proteins previously characterized as components of a ROS detoxification pathway or as part of an oxygen responsive gene cluster (36, 60). These namely include reverse rubrerythrins (*rbr3A/B*), desulfoferrodoxin (*dfx*), rubredoxin (*rd*), NADH dependent rubredoxin oxidoreductase (NROR) and the oxygen reducing flavodiiron proteins FprA1 and FprA2 (Table 2). High expression of these genes represented an efficient indicator that the global transcriptional changes observed resulted from intense oxidative stress as result of O_2 exposure. Other strongly activated genes which products play central role in the cell's redox balance included glutaredoxin and thioredoxins dependent systems (Table 2). The relatively high number of homologues and associated peroxidases suggests a vital role during oxidative stress. Interestingly, significant transcriptional changes under these conditions were not observed for the two annotated superoxide dismutases from the Cu/Zn (*CAC2567*) and Fe/Mn type (*CAC1363*). Furthermore the transcription of the rubrerythrins Rbr1 and Rbr2, which are characterized by their N-terminal ferritin-like domains, and the rubredoxin-like domains on the C-terminus did also not respond to the presence of O_2 (Table 2).

Nucleic acid repair and iron uptake. Anaerobes in particular are highly susceptible to oxidative damage to proteins and DNA via direct O_2 dependent inactivation of Fe-S clusters or Fenton type chemistry (30). A more detailed view on the oxygen stimulon revealed that *C. acetobutylicum* not only induces active detoxification of oxygen and reactive derivatives but also prompts the repair of essential celluar components. The repair of damaged DNA is activated by an induced transcription of genes encoding enzymes involved in the *de novo* synthesis of nucleotides, e. g. enzymes of the purine metabolism (*CAC1390-95, CAC1655*), and three ribonucleotide diphosphate reductases (*CAC1047, CAC3276/7*), as well as those responsible for excision and replacement (*uvrA/B, CAC0502/3*) (Table 2). As expected from the conflictive role of iron as cofactor in defence enzymes and catalyst in Fenton chemistry iron uptake genes were strictly regulated under oxygen. Two Feo-type uptake systems for Fe^{2+} and one ferrichrome dependent system were upregulated (*CAC0447/8, CAC0788-91, CAC1029-32*), which could support the induced Suf and Nif machinery (*CAC3288-92*) in the biogenesis of Fe-S clusters. Under the same conditions a large operon (*CAC1988-94*) putatively involved in the uptake of Fe^{3+} and molybdenum cofactor synthesis was repressed (Table 2). Reduced iron might become scarce due to an increasing production of reverse rubrerythrins and flavodiironproteins, but at this point the differential regulation of Fe^{2+} and Fe^{3+} cannot be fully explained. Previous studies with *E. coli* have shown that *moa* genes for molybdenum cofactor production are enhanced under anaerobic conditions and underlie the control of the anaerobic activator protein FNR (1, 65). Molybdenum cofactors are known to catalyze reactions carbon, sulphur, and nitrogen cycles and act as sites of substrate binding and reduction in nitrogenases (39, 64).

TABLE 2. Relative transcript levels of selected genes of wild type *C. acetobutylicum* during oxygen exposure (O_2) and of anaerobic *C. acetobutylicum* lacking PerR (PerR$^-$).

ORF# *	gene	protein function	COG class	fold regulation	
				O_2	PerR$^-$
detoxification / redox balance					
CAC0327	*bcp*	bacterioferritin comigratory protein, akylhydroperoxidase	O	5.2	16.0
CAC0869		thioredoxin reductase	O	4.9	8.1
CAC1027	*fprA1*	flavoprotein FprA1	C	4.4	16.1
CAC1363	*sodC*	superoxide dismutase. Cu-Zn family	P	-	0.21
CAC1547	*trxA*	thioredoxin	O	7.5	-
CAC1548	*trxB*	thioredoxin reductase	O	10.3	-
CAC1549	*bsaA1*	glutathione peroxidase	O	23.3	-
CAC1570	*bsaA2*	glutathione peroxidase	O	2.6	0.1
CAC1571	*bsaA3*	glutathione peroxidase	O	5.1	-
CAC2448	*nror*	NADH dependent rubredoxin oxidoreductase, NROR	C	7.8	16.8
CAC2449	*fprA2*	flavoprotein FprA2	C	7.3	22.5
CAC2450	*dfx*	desulfoferrodoxin, superoxide reductase, SOR	C	18.4	29.6
CAC2567	*sod*	superoxide dismutase. Fe-Mn family	P	-	-
CAC2575	*rbr1*	rubrerythrin	C	-	0.42
CAC2777		glutaredoxin	O	3.5	6.0
CAC2778	*rd*	rubredoxin	C	5.2	14.4
CAC3018	*rbr2*	rubrerythrin		-	-
CAC3306	*tpx*	thiolperoxidase	O	3.0	3.4
CAC3597	*rbr3B*	reverse rubrerythrin	C	5.6	26.9
iron metabolism /transport					
CAC0447	*feoA1*	FeoA protein. involved in Fe^{2+} transport	P	2.3	-
CAC0448	*feoB2*	Fe^{2+} transport protein B (feoB-2)	P	2.8	-
CAC0788		Ferrichrome transport permease	P	3.3	
CAC0789	*fhuB*	permease	P	3.4	
CAC0790	*fhuD*	ferrichrome-binding periplasmic protein	P	2.6	-
CAC0791	*fhuC*	ferrichrome ABC transporter. ATP-binding protein	P	2.3	-
CAC1029		FeoA-like protein. involved in iron transport	P	5.8	-
CAC1030		FeoA-like protein. involved in iron transport	P	8.1	-
CAC1031		FeoB-like GTPase. responsible for iron uptake	P	15.9	-
CAC1032		predicted transcriptional regulator	K	4.3	-
CAC1988		ferrichrome-binding periplasmic protein	P	0.27	-
CAC1989		ABC-type Fe^{3+} transport system, ATPase component	P	0.27	-
CAC1990		ABC-type Fe^{3+} transport system, permease component	P	0.30	-
CAC1991		uncharacterized protein, YIIM family	S	0.32	-
CAC1992	*moaC*	Mo cofactor biosynthesis protein C	H	0.30	-
CAC1993	*moaA*	Mo cofactor biosynthesis protein A	H	0.30	-
CAC1994	*moaB*	molybdopterin biosynthesis enzyme	H	0.32	-
CAC1995		hypothetical protein	X	0.31	-
CAC1996		hypothetical protein	X	0.36	-
nucleic acid repair / metabolism					
CAC0502	*uvrB*	excinuclease ABC subunit B	L	2.1	-
CAC0503	*uvrA*	excinuclease ABC subunit A (ATP-ase)	L	4.2	-
CAC1047		ribonucleotide-diphosphate reductase α subunit	F	9.9	-
CAC1390	*purE*	phosphoribosylaminoimidazole carboxylase catalytic subunit	F	3.2	-
CAC1391	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase	F	3.1	-

CAC1392	*purF*	amidophosphoribosyltransferase	F	2.8	-
CAC1393	*purM*	phosphoribosylaminoimidazole synthetase	F	2.9	-
CAC1394	*purN*	phosphoribosylglycinamide formyltransferase	F	2.8	-
CAC1395	*purH*	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	F	2.5	-
CAC1655	*purQ/L*	bifunctional phosphoribosylformylglycinamidine synthase	F	3.5	-
CAC1815	*recA*	recombinase A	L	4.0	-
CAC1856		ribonuclease HI	R	2.4	4.4
CAC3276	*nrdB*	ribonucleotide-diphosphate reductase β subunit	F	13.5	-
CAC3277	*nrdA*	ribonucleotide-diphosphate reductase α subunit	F	6.5	-
CAC3358	*dam*	site-specific DNA methylase	L	6.4	
CAC3499		predicted endonuclease	G	5.3	4.1
protein turnover / repair					
CAC1857		metal dependent peptidase	S	2.2	4.8
CAC3288	*sufC*	Fe regulated ABC transporter ATPase subunit	O	2.8	-
CAC3289	*sufB1*	Fe regulated ABC-type transporter membrane component	O	2.9	-
CAC3290	*sufB2*	Fe regulated ABC-type transporter membrane component	O	3.3	-
CAC3291		selenocysteine lyase, NifS family	E	2.6	-
CAC3292		NifU homolog involved in Fe-S cluster formation	C	2.0	-
CAC3624		6-pyruvoyl-tetrahydropterin synthase	H	8.2	-
CAC3625		Fe-S oxidoreductase of MoaA family	O	3.1	-
energy conversion					
CAP0162	*adhE1*	aldehyde dehydrogenase (NAD^+)	C	1.9	2.2
CAP0163	*ctfa*	butyrate-acetoacetate COA-transferase subunit A	C	1.9	2.1
CAP0164	*ctfb*	butyrate-acetoacetate COA-transferase subunit B	C	2.0	2.2
CAC0116		carbonmonoxide dehydrogenase. beta chain	C	23.9	0.05
CAC0587		flavodoxin	C	11.4	-
CAC0711	*tpi*	triosephosphate isomerase	G	2.1	3.2
CAC0712	*pgm*	phosphoglyceromutase	G	2.8	9.0
CAC0713	*eno*	phosphopyruvate hydratase	G	3.3	6.9
CAC2452		flavodoxin	C	3.6	11.6
CAC2458		2-oxoglutarate ferredoxin oxidoreductase, β-subunit	C	9.3	40.0
CAC2459		2-oxoacid ferredoxin oxidoreductase, α-subunit	C	9.1	23.8
CAC2499		pyruvate ferredoxin oxidoreductase	C	-	0.3
CAC2708	*hbd*	3-hydroxybutyryl-CoA dehydrogenase	I	0.34	-
CAC2709	*etfA*	electron transfer flavoprotein α-subunit	C	0.29	-
CAC2710	*etfB*	electron transfer flavoprotein β-subunit	C	0.26	-
CAC2711	*bcd*	butyryl-CoA dehydrogenase	I	0.28	-
CAC2712	*crt*	enoyl-CoA hydratase, crotonase	I	0.28	-
CAC2873	*thlA*	thiolase	I	0.31	-
CAC3075	*buk*	butyrate kinase	C	0.41	-
CAC3076	*ptb*	phosphate acetyltransferase	C	0.35	-
CAC3657		NADP-glyceraldehyde-3-phosphate dehydrogenase	C	19.0	26.7
CAC3658		conserved membrane protein, SapB/MtgC family	S	4.0	12.5
CAC3659		S-adenosylmethionine-dependent methyltransferase	Q	2.1	4.7
arginine biosynthesis					
CAC0316	*argF/I*	ornithine carbomoyltransferase	E	-	0.15
CAC0376		N-dimethylarginine dimethylaminohydrolase	E	2.4	2.1
CAC0973	*argG*	argininosuccinate synthase	E	-	0.12
CAC0974	*argH*	argininosuccinate lyase	E	-	0.27
CAC2388	*argD*	N-acetylornithine aminotransferase	E	-	0.11
CAC2389	*argB*	acetylglutamate kinase	E	-	0.18
CAC2390	*argC*	N-acetyl-γ-glutamyl-phosphate reductase	E	-	0.29
CAC2391	*argJ*	ornithine acetyltransferase/N-acetylglutamate synthase	E	-	0.31

[a] Brackets indicate genes that presumably form a transcriptional unit.

Enzymes involved in central metabolism and energy conversion. An O_2 sensitive pyruvate-ferredoxin oxidoreductase, encoded by *CAC2229* and *CAC2499*, is the central metabolic enzyme in *C. acetobutylicum* (48). Interestingly, the transcription of genes encoding the two subunits of an alternative α-ketoacid ferredoxin oxidoreductase (*CAC2458/9*) are highly induced under oxidative conditions (Table 2). The clostridial enzyme has not been studied thus far, but studies on related enzymes from the aerobic archea *Sulfolobus sp.* strain 7 and *Aeropyrum pernix* have demonstrated a wide substrate spectrum for ketoacids and even more importantly aerobic stability (51, 72). It is thus not unlikely that an oxygen-labile PFOR is replaced by an oxygen resistant enzyme to feed the pool of acetyl CoA. The megaplasmid encoded *sol* operon (*CAP0162-4*) was slightly upregulated under O_2 (Table 2). Its protein products are thought to be involved in the early phase of solvent formation (14). Essentially all other genes which protein are assigned to function downstream of acetyl CoA in the formation of butyrate are moderately downregulated (2-5fold) under these conditions. There are two other enzymes which are activated by O_2 and for which a central role in carbon metabolism is apparent (Table 2): One is an NADP dependent functional homologue of the glycerinaldehyd-3-phophate dehydrogenase (GAPDH), which will supply NADPH for the cell's antioxidant system. Supporting its functional role during glycolysis, the genes encoding the enzymes responsible for other reactions in this pathway (triosephophatisomerase, phosphoglyceromutase, enolase; *CAC0711-13*) are also moderately upregulated under O_2 (Table 2). The other highly activated gene translates into a protein which belongs to the Ni containing carbon monoxide dehydrogenase (CODH) family which includes actual CO dehydrogenases and acetyl CoA synthases of methanogenic and acetogenic organisms (11). When comparing the amino acid sequence of the oxygen induced proteins from *C. acetobutylicum* to the known homologues of *Carboxydothermus hydrogenoformans*, the clostridial peptide shows the lowest similarity to the acetyl CoA synthase subunits. Furthermore the absence of genes encoding any other subunit of acetyl CoA synthases makes it rather unlikely that this protein is involved in CO_2 dependent acetyl CoA formation. The highest amino acid identity scores with 32%, and 30% are found in comparison to the least described CODHs, CooSV and CooSIV, respectively (data not shown). Interestingly, *C. hydrogenoformans* the later one is encoded in an operon with two central components of anaerobic ROS detoxification, namely rubrerythrin and NROR. These findings have lead to the speculation that in *C. hydrogenoformans* CooSIV might transfer electrons from CO to rubrerythrin via NROR (71).

The PerR regulon of *C. acetobutylicum*. A peroxide repressor-like (PerR) protein has recently been shown to act as a central regulator of the O_2 detoxification system of *C. acetobutylicum*, as full activation of its regulon triggered aerobic survival and even limited growth (25). To find targets of PerR regulation and gain insight into the gene expression profile of an aerotolerant strain the global transcription in *perR* deleted cells and cells carrying the wild type allele was compared. The complete absence of PerR resulted in a highly differential expression (>3fold) of

145 genes. Of these, 51 genes were upregulated in the *perR* deleted strain, which overlapped the oxygen stimulon of the wild type strain. Nearly half of the genes (22 ORFs) which were induced in *perR* deleted cells were also found as highly expressed when the wild type was exposed to oxygen for one hour, while the remaining 29 genes were exclusively activated in the mutant strain (Fig. 1B). All differentially expressed genes (>2 fold change in transcription) of the PerR mutant were also assigned to functional categories using the cluster of orthologous groups (COG). Similar to the results obtained for the O_2 stimulon, the majority of differentially expressed genes (37 % of the upregulated genes and 51 % of the downregulated genes) encoded proteins which either could not be grouped or were of unknown function (X). Among the others, those involved in energy production and conversion (C, 8.4 % of the upregulated genes, and 5 % of the downregulated genes), amino acid transport and metabolism (E, 7.9 %, and 11 %, respectively) and transcription (K, 8.4 %, and 2 %, respectively) were highly represented in *C. acetobutylicum* lacking PerR (Fig. 1A). The high number of 93 genes which were strictly downregulated (>3fold) in the mutant demonstrated that PerR is essential for the expression of certain genes and/or might also have an activating role during anaerobic growth. Supporting these ideas, none of the lower expressed genes in the mutant were found as lesser transcribed during oxygen exposure of the wild type (Fig. 1B). Hence, the downregulation of genes in the *perR* mutant was apparently rather independent of the cells oxygen response. There is only two exceptions to this point which deserve some attention: The gene encoding the carbonmonoxide dehydrogenase like protein (CODH, *CAC0116*) was highly activated when wild type cells are aerated, but transcription of this gene is most strongly repressed in the $PerR^-$ strain (Table 2). Similarly, the gene encoding the gluthione peroxidase suggesting that an inactivated state of the PerR protein might be required in order to obtain full expression of *CAC0116* under oxygen.

PerR represses O_2 and ROS reduction. A first study on the role of PerR in *C. acetobutylicum* identified a flavodoxin (*CAC2452*), the flavodiironprotein FprA1 (*CAC1027*), and most prominently the twin gene encoded reverse rubrerythrin Rbr3A/B (*CAC3597/8*) as highly expressed in the absence of PerR (25). The transcripts of all these proteins were among the most dramatically induced ones. Highest expression values in the *perR* deleted strain were determined for the genes and operons encoding Rbr3A/B (*CAC3597/8*), FprA1 (*CAC1027*) glutaredoxin (*CAC2777*), and NROR-FprA2-Dfx (*CAC2448-50*). Fluorescence counts for these transcripts exceeded those for elongation factor Tu by a factor of up to 4.3 in the case of Rbr3A/B which was equivalent to saturation (>80.000) even when using high resolution scans (Table S2). Therefore the induction of these highly abundant transcripts might be underestimated (Table 2). Essentially all other genes previously reported as members of anaerobic ROS detoxification or redox balance were also activated in the mutant, e.g. rubredoxin (*CAC2778*), the alkylhydroperoxidase Bcp (*CAC3027*) and a thiolperoxidase (*CAC3306*). Their induction in the absence of PerR even during anaerobiosis, suggested that

PerR is the key regulator which represses these proteins during anaerobic growth of the wild type. Interestingly, the glutathione and thioredoxin antioxidoant system, though activated by oxygen, seems to depend on an alternative regulatory mechanism as no significant alterations in their transcript levels were detected in PerR$^-$ (Table 2). Similar observations were made for the genes involved in nucleic acid repair or iron metabolism/transport, demonstrating that PerR is not the sole level of regulation and yet unidentified regulatory circuits exist. Only few genes of the central carbon pathways were differentially expressed in the *perR* mutant. These include the oxo-acid ferredoxin oxidoreductase operon (*CAC2458/9*), the NADP dependent GAPDH (*CAC3657*) and the enzymes which catalyze the following two glycolytic reactions. The gene for the NADPH dependent GAPDH might form an operon with two genes encoding an unknown membrane protein and a possible S-adenosylmethionine-dependent methyltransferase (CAC3658/9) as both of these genes were also induced (Table 2). A total of 9 genes were strictly repressed (>10 fold lower expression) in the mutant strain and hence, strongly dependent on the presence of a functional PerR protein for expression. These included a gene (*CAC0553*) and an operon (*CAC0056-59*) of unknown function, two genes with a putative role in the biosynthesis or transport of siderophores (*CAP0029/30*), the gluathione peroxidase BsaA2 (*CAC1571*) and the CODH (*CAC0116*). Only the latter was highly O_2 inducible in wildtype cells. Many genes encoding enzymes for the biosynthesis of arginine were moderately downregulated in the mutant, which suggest that arginine could function as a source of intracelluar nitric oxide (NO) in *C. acetobutylicum*. Consequently, enzymes involved in the inhibition of NO production (N-dimethylarginine dimethylaminohydrolase, *CAC0376*) and FDPs as reductive NO scavengers (26) are upregulated in PerR$^-$ and in O_2 exposed wild type cells. In summary PerR acts primarily as a repressor of an O_2 responsive, large scale ROS defense machinery, but also targets those O_2 sensitive bottle necks which might be crucial for a continuous demand of NAD(P)H and ATP.

Direct targets of PerR repression. An earlier study on the stress dependent transcription of reverse rubrerythrins from *C. acetobutylicum* identified an inverted repeat of 24 nucleotides (IR1) in the 5' untranslated region (5'UTR) of *rbr3A* for which a regulatory function was proposed (24). When reversed, this palindromic sequence shares high similarity with to the known consensus binding sequences of the peroxide repressors PerR from *B. subtilis* and *Staphylococcus aureus* (Fig 2a). In the closely related facultative aerobe *B. subtilis* PerR derepresses transcription of multiple target genes upon its metal catalyzed inactivation with H_2O_2 (40).To further support the idea that IR1 was the site of PerR regulation and to identify other potential target genes a genome wide search for IR1 was performed using the "Virtual footprint" software (50). When using exclusively the palindromic nucleotides and allowing a single mismatch a total of ten genes was found that carried a potential PerR box within 200 bp of their start codon (Table 3). Nine of them were amongs those with the highest induction in the *perR* mutant, including the ones encoding FprA1, the entire O_2 responsive operon with NROR,

FprA2, and Dfx, the glutaredoxin-rubredoxin operon, and both subunits of an oxo-acid ferredoxin oxidoreductase (Table 3). The gene encoding the NADP dependent GAPDH which was also highly expressed under oxygen and in the absence of PerR was preceded by a PerR box with 3 mismatches. Several conserved nucleotide positions were detected for this regulatory module which could be illustrated as a sequence logo (Fig. 2b).

TABLE 3. Regulation of genes with PerR boxes during oxygen exposure (O_2) and of anaerobic *C. acetobutylicum* lacking PerR (PerR$^-$).

ORF#	gene/ operon	function	sequence motif		fold regulation	
					O_2	PerR$^-$
CAC0327	*bcp*	akylhydroperoxidase	TATtttTATTAtaTAATAataATT	(-17)	5.2	16.0
CAC0421		hypothetical protein	ATTaatTATTAtaTAATAtttATT	(-34)	2.3	2.6
CAC0491		hypothetical protein	AATttcTATTActTAATTaatATT	(-15)	-	-
CAC0756/ CAC0757		multimeric flavodoxin WrbA / predicted membrane protein	AATttaTATTAttTAAAAtccATT	(-112) (-74)	- -	- -
CAC1027	*fprA1*	flavoprotein	AATaatTATTAaaTAATAtaaATT	(-180)	4.4	16.1
CAC2448/ CAC2449/ CAC2450	*nror- fprA2- dfx*	NADH-rubredoxin oxidoreductase flavoprotein FprA2, and SOR	AATaatTATTAgtTAATAtaaATT	(-153)	7.8 7.3 18.4	16.8 22.5 29.6
CAC2452	*flx*	Flavodoxin	AATaatTATTAt TAATTaacTTC	(-18)	3.6	11.6
CAC2459/ CAC2458		2-oxoacid:ferredoxin oxidoreductase α / β-subunit	AATaaaTATTAttTAATAattAAT	(-89)	9.3 9.1	40.0 23.8
CAC2634	*perR*	peroxide repressor	TATgtaTATTAtaTAATAaaaATT	(-46)	2.6	n.d.
CAC2777/ CAC2778	*grx rd*	glutaredoxin rubredoxin	AATtaaTATTAatTAATAattGTT	(-42)	3.5 5.2	6.0 14.4
CAC3306	*trx*	thiolperoxidase	AATagaTATTActTAATAataAAT	(-159)	3.0	3.4
CAC3598/ CAC3597	*rbr3A- rbr3B*	reverse rubrerythrins	AATaatTATTAtaTAATAacaATT	(-109)	5.6	26.9
CAC3657		NADP dep. GAPDH	AACgatTATTAaaAAATAataACT	(-137)	19.0	26.7
CAC3659		S-adenosylmethionine-dep. methyltransferase	TAGattTATTAtaTAATAaaaATA	(-47)	2.1	4.7

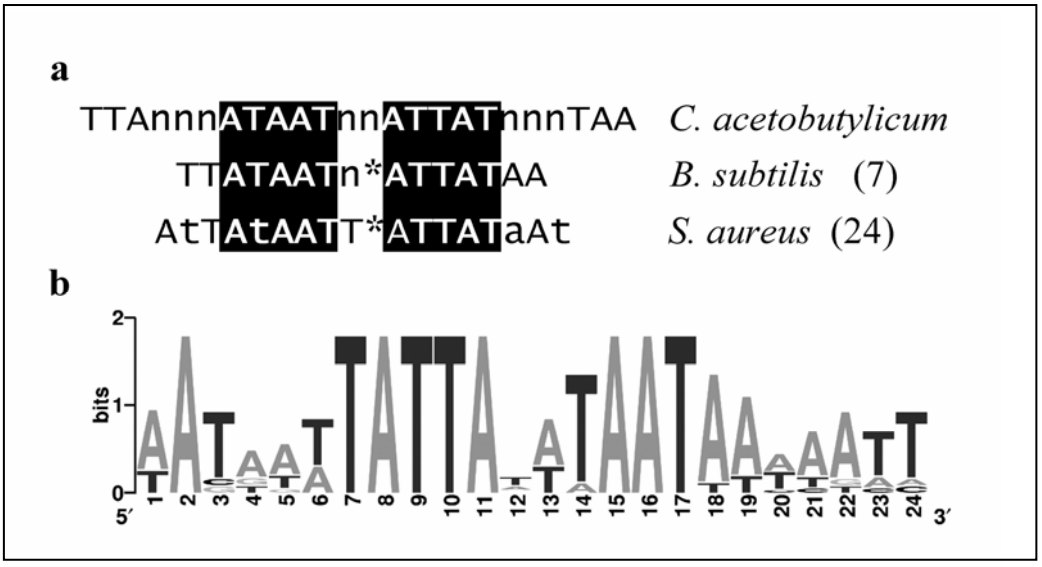

FIG. 2. A PerR consensus-like conserved sequence motif in *C. acetobutylicum*.

(a) Aligment of the reversed IR1 from *C. acetobutylicum* (24) to known PerR boxes from *B. subtilis and S. aureus*.

(b) Sequence logo representing conserved positions in the *C. acetobutylicum* PerR box as identified upstream of common-responsive genes (see Table 3).

PerR binds to the *rbr3A/B* promoter region. To give evidence that the clostridial PerR homologue directly interacts with the promoter region of *rbr3A* its gene was heterologously overexpressed in *E. coli*. The PerR encoding ORF *CAC2634* was cloned in the pT plasmid to obtain a fusion protein with an N-terminal Strep-Tag. Gelelectrophoresis of the purified fraction yielded two distinct bands which were in agreement with the different oxidation states observed for the *B. subtilis* peptide (41, Fig. 3a). The purified protein was incubated with a labelled DNA fragment of the *rbr3A* promoter region covering the last 235 bp of the 5'UTR and IR1. When these reactions were applied to gelelectrophoresis the mobility of this fragment was reduced, depending on the amount of PerR in the assay (Fig 3b, lane 1-3). This shift in DNA mobility could be reversed stepwise following the addition of unlabeled fragment giving evidence that the binding of PerR was DNA specific (Fig. 3b, lane 4-5).

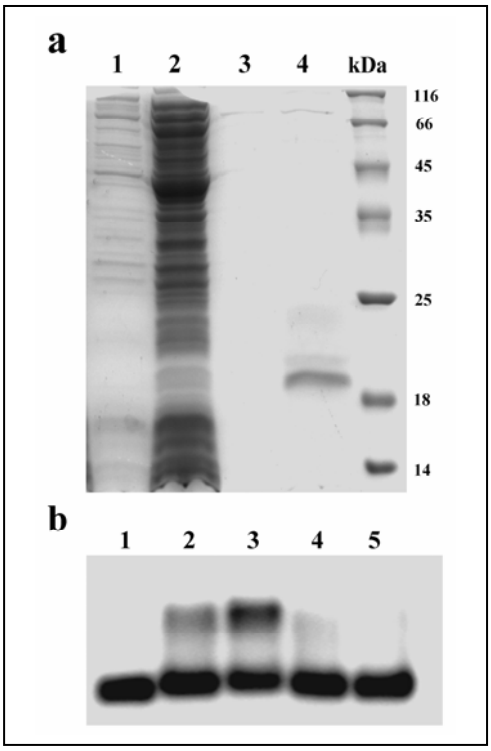

FIG. 3. Interaction of purified PerR with the *rbr3A/B* promoter region.

(a) Coomassie stained SDS-PAGE (12.5%), 1+2: 10 and 50 µg of crude extract from *E. coli* expressing PerR of *C. acetobutylicum*; 3+4: purfication fractions; kDa: molecular weight marker.

(b) Electrophoretic mobility shift assays were carried out with 12.5 nM of the *rbr3A/B* promoter fragment. Fragments were incubated without protein (lane 1), or with 35 nM (lane 2) and 50 nM (lane 3-5) of purified PerR. As negative controls, 25 nM (lane 4) or 50 nM (lane 5) of an unlabelled promoter fragment were also added to the reactions.

Hydrogen peroxide acts as an indirect signal for oxygen. The conserved binding motif and interaction with the promoter region of *rbr3A/B* suggested that the mode of PerR regulation is conserved in *C. acetobutylicum*, albeit with different target genes when compared to facultative aerobes. To monitor PerR binding *in vivo* and give evidence that rather H_2O_2 than O_2 is used as a sensor molecule during aeration complementational studies were performed. The clostridial PerR protein was expressed from a xylose inducible promoter in *B. subtilis* strains lacking either the ferric uptake regulator (Fur) or PerR. Subsequently, their sensitivity towards H_2O_2 was monitored in a zone of inhibition assay (Fig. 4). In the presence of xylose the wild type *B. subtilis* strain expressing $PerR_{Cac}$ differed only marginally from its parent in its sensitivity towards H_2O_2, which might reflect the higher affinity of $PerR_{Bsu}$ for its own binding site (Fig. 4a+b). No effect on the sensitivity towards H_2O_2 was seen when *B. subtilis* cells were lacking the Ferric uptake regulator Fur, which is in agreement with earlier results for logarithmic cells (6, Fig. 4c). However, low concentrations of H_2O_2 peroxide facilitated growth of the *fur* mutant on iron rich medium, presumably due to induction of the cells peroxide stimulon which might partially overcome oxidative stress as a result of high intracellular iron levels. Unexpectedly induced expression of the clostridial peptide also has a stimulating effect on the growth of *B. subtilis fur* mutant even in the absence of H_2O_2 (Fig. 4d). At this point it is not fully clear whether this effect is specific for the clostridial protein, but the Bacillus Fur and PerR proteins were shown to control exclusive regulons (2, 21). *B. subtilis* cells lacking their own perR gene are hyperresistent towards H_2O_2 due to an increased production of defence proteins (6). Following the expression of $PerR_{Cac}$ this hyperesistant phenotype was converted to a

hypersensitive one, giving evidence that the protein from *C. acetobutylicum* can indeed acts as a repressor despite aerobic growth (Fig. 4e+f). The peroxide sensitivities of all strains were largely reflected by their catalase activities. Wild type cells showed normal levels of catalase that were even moderately reduced when expressing $PerR_{Cac}$, but catalase was nearly 100fold more active in a perR deleted strain (Fig. 5). When in turn the clostridial peptide was produced in the cells, catalase acitivity was reduced by more than 200fold to levels far below those of wild type cells (Fig. 5). Consecutive low doses of H_2O_2 were able to partially restored catalase activity (data not shown), giving evidence that the key regulator of oxygen defence in *C. acetobutylicum* is not susceptible to molecular O_2, but rather senses intracellular peroxide levels.

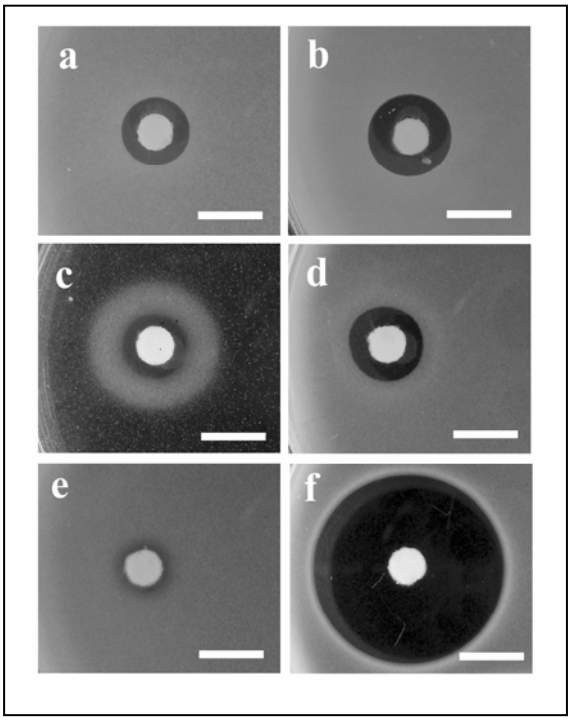

FIG. 4. Zone of inhibiton assay for different *B. subtilis* strains with H_2O_2. Midlogarithmic cells were added to prewarmed LB-agar, poured into Petri dishes and a disk with 5 µl of 1 M H_2O_2 were placed on the agarplates. Growth and zones of inhibition for each strain were monitored following an overnight incubation at 30°C. White bars indicate 10 mm. **(a)** HB1000, wild type **(b)** $HB1000R_{Cac}$, wild type expressing $PerR_{Cac}$ **(c)** HB6543, *fur*$^-$ **(d)** $H6543R_{Cac}$ *fur*$^-$ expressing $PerR_{Cac}$ **(e)** HB0509, *perR*$_{Bsu}^-$ **(f)** $HB0509R_{Cac}$, *perR*$_{Bsu}^-$ expressing $PerR_{Cac}$.

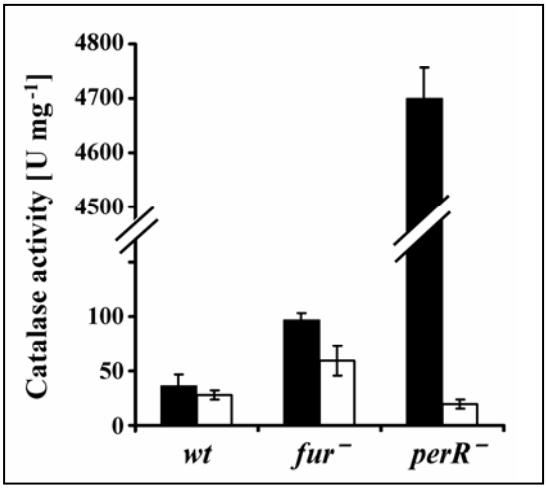

FIG. 5. Catalase activities in $PerR_{Cac}$ complemented *B. subtilis.* Catalase was measured in crude extracts from mutant cells of *B. subtilis* (black columns) or from complemented mutant cells expressing PerR of *C. acetobutylicum* (white columns). Names below the columns indicate the relevant *B. subtilis* genotypes, which were either wild type (*wt*), *fur*- (*fur*$^-$), or *perR* mutants (*perR*$^-$).

DISCUSSION

The finding that oxygen sensitive enzymes are widely used as central components in the metabolism of anaerobes, as well as the existence of a robust defence system has lead to a refinement of the traditional model of obligate anaerobiosis. An energy metabolism which exclusively relies on fermentation of highly reduced substrates demands enzymes with low redox potentials and thus, an environment which is predominantly anaerobic. *C. acetobutylicum* flourishes under fully anaerobic conditions, and even maintains growth at a reduced rate when the influx of oxygen is compensated by its continuous reduction (35). Only when dissolved oxygen accumulates, central metabolic enzymes like PFOR are wrecked at rate that metabolism is halted and viability decreases (48). In a natural environment exposure to O_2 might occur gradually and thus, allow an efficient adaptation (31). This has just recently been shown for a number of other bacteria from the diverse group of anaerobes, e. g. growth of sulphate reducing *D. vulgaris* was only marginally effected when exposed to 0.1 % (v/v) O_2 and the hyperthermophile *Thermotoga maritima* was able to grow even if 0.5 % (v/v) of O_2 were added to the growth phase (43, 49). The experimental setup presented here was designed that the influx of O_2 was low enough to assure metabolic activity but sufficient to generate substantial cellular damage, which was visible as a reduced growth rate. Microaerobic growth as well as deletion of a central regulator in oxidative stress resulted in global changes in gene expression under O_2 leading to a preliminary model on how *C. acetobutylicum* attempts to combine O_2/ROS removal, damage repair and adjustments in central energy metabolism to avoid paralysis (Fig. 6).

The production of proteins involved in detoxification and cellular redox balance was most drastically activated, which was expected as upregulation of these components has been observed recently in a number of bacteria which are considered as anaerobes (43, 49, 68). The massive expression of all components of the proposed oxygen and ROS detoxification system which involves FDPs, Dfx, NROR, rubredoxin, and reverse rubrerythrins emphasized its vital role in survival. Consequently, all of these components were found be the primary targets of PerR repression, as they were overexpressed in the *perR* deleted strain and shared a common regulatory motif. Thus, it seems likely that PerR acts as a hypersensitive regulator designed to activate these genes even in the presence of traces of oxygen. The acute induction of this system in a natural environment will also support the restoration of anaerobiosis by a complete uptake of residual oxygen.

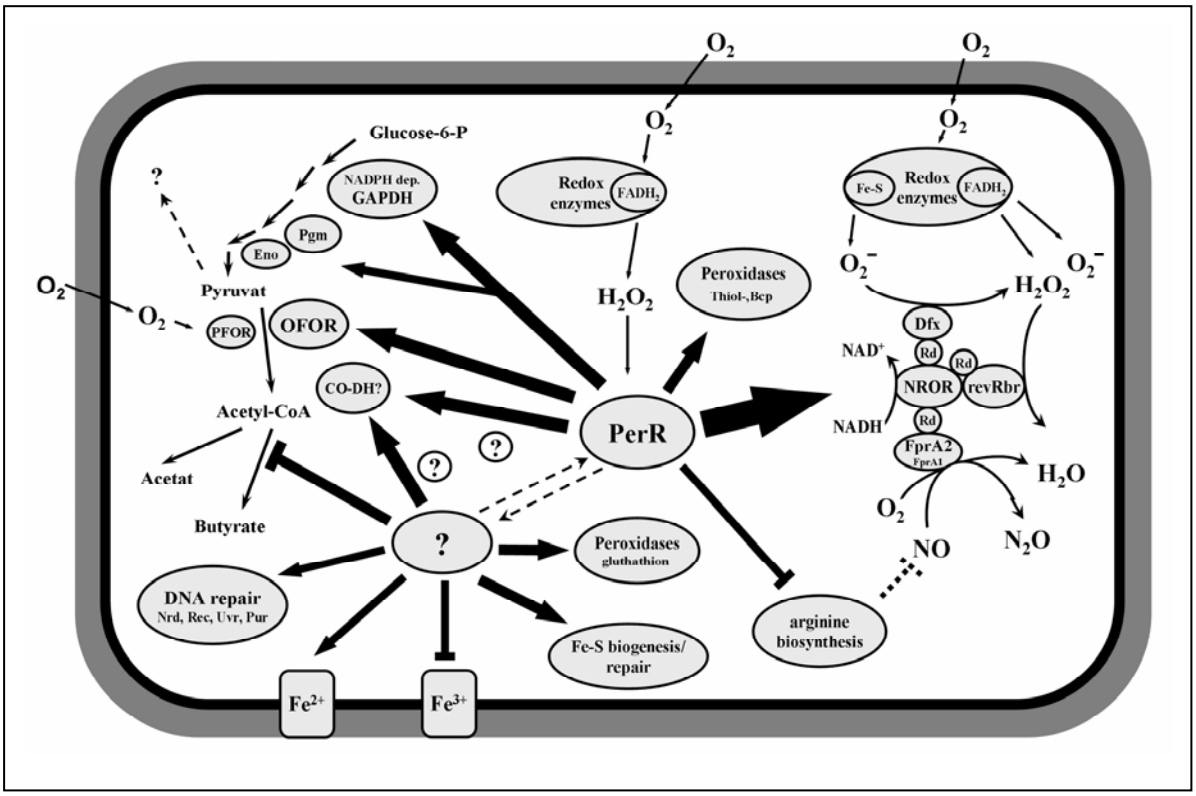

FIG. 6. Model of the transcriptional response to O_2 of *C. acetobutylicum*.

Differentially regulated genes are represented as encoded proteins or as functional groups and are highlighted in grey. Abbreviations used are the same as in Table 2. Arrows indicate up- (normal) or downregulation (blunt) at direct or indirect regulatory targets and their relative expression under O_2.

The PFOR could be a major bottleneck in glucose degradation and acetyl CoA production under microaerobic conditions. An enzyme with higher resistance towards oxidative inactivation would clearly be of advantage during oxygen exposure. At this time the induced oxoacid-ferredoxin oxidoreductase (OFOR) has not been functionally characterized but the use of similar enzymes in aerobic archea make it tempting to speculate that a replacement occurs under these conditions (51, 72). Furthermore, *C. acetobutylicum* attempts to reload the pool of NADPH by inducing an alternative GAPDH which was shown to be an NADP dependent enzyme in *E. coli* (45). The reduced form of this nucleotide is the driving force in the maintenance of a reduced interior, e.g. by acting as a cofactor of gluthatione reductase (20, 53). Interestingly, OFOR and NADP dependent GAPDH seem to be two of very few proteins that function outside the detoxification system and are still subject to direct PerR repression. This might indicate their significance during the early response to oxygen, while glutathione and thioredoxin dependent rescue systems were PerR independent and could belong to circuits that protect from a persistent exposure to oxidative conditions. Similar mechanisms can be postulated for nearly all metabolic enzymes with altered expression under O_2. In an oxidizing

environment *C. acetobutylicum* seems to direct its electron flow towards the higher oxidized product acetate. This is in agreement with a slightly increased acetate production by *C. acetobutylicum* under O_2 which has been observed during an earlier investigation (54). The results presented here demonstrate that essentially all genes in the formation of butyrate downstream from acetyl CoA are downregulated suggesting that this pathway is less favourable when sufficient levels of NAD(P) are regenerated during the reduction of O_2 and ROS. The moderate upregulation of the *sol* operon integrates in the larger general stress network finally inducing solvent formation and sporulation. Its exact contribution to solvent production as well as its regulation has not been fully established (13). Thus, it cannot be ruled that at least the elevated expression of the CoA transferase subunits CtfA/B could support the formation of acetyl CoA which is an important intermediate in membrane synthesis and therefore a crucial factor for cell division.

Another enzyme with highly activated expression under O_2 is a member of the CO dehydrogenase protein family. A role for these proteins in the oxidative stress response has not been established but a homologue from *C. hydrogenoformans* was speculated to function in ROS detoxification due to its genomic position in an operon with rubrerythrin and NROR (71). Furthermore, it cannot be excluded a this point that the clostridial protein also serves a protective function in hydrogen production of this organism. The H_2 evolving hydrogenaseA1 (HydA1) of *C. acetobutylicum* is highly sensitive to oxygen dependent inactivation (10, 18). While low doses of molecular O_2 lead to irreversibly damaged enzymes, CO has been shown to act as a competitive inhibitor that antagonizes O_2 and can greatly reduce the hydrogenase inactivation state (15, 44). The generation of CO via reduction from CO_2 could therefore prevent irreversible wreckage during short exposures to oxygen. The completely opposite expression patterns of this protein in both strains partially support this idea: The gene encoding the CODH showed high activation when the wild type was exposed to O_2, but lowest transcription in anaerobic *perR* deleted cells. The opposed expression of this gene in both strains addresses another interesting issue of PerR regulation. It does not seem unlikely that PerR could also function as a gene activator in its oxidized form via a yet undefined mechanism. Direct Gene activation by PerR has already been seen for the *B. subtilis srfA* operon (19). ROS induced transcriptional activation, though previously assigned to SoxR, OxyR, has more recently been demonstrated for the *Borellia* oxidative stress regulator BosR, a homologue of the *B. subtilis* PerR (3, 23, 74).

The regulatory role in the peroxide stimulon as well as the mechanism of inactivation by H_2O_2 in a Fenton type reaction have been thoroughly described for the *B. subtilis* peptide (6, 17, 22, 40). In the anaerobe *C. acetobutylicum* external addition of H_2O_2 was not required to activate the organisms PerR regulon, while even low doses of O_2 were an adequate stimulus. Complementation of a *B. subtilis* strain lacking the two Fur proteins (PerR and the ferric uptake regulator Fur itself) with $PerR_{Cac}$ provided evidence that the clostridial protein was a fully functional homologue of PerR *in vivo*. The presence of the clostridial peptide in a strain lacking

$PerR_{Bsu}$ repressed the expression of oxidative stress proteins during aerobic growth, and resulted in a peroxide hypersensitive phenotype. Low doses of external H_2O_2 inactivated $PerR_{Cac}$ and derepressed catalase. However, as indicated from the proposed regulatory sequence motif in *C. acetobutylicum*, the specificity of $PerR_{Cac}$ for the *B. subtilis* PerR boxes seems to be reduced. When $PerR_{Cac}$ was expressed in wild type *B. subtilis* cells only little increase in H_2O_2 sensitivity was determined, presumably as a result of preferred binding of $PerR_{Bsu}$. Nearly the same effect could be observed when $PerR_{Cac}$ was substituted for the *Bacillus* Fur protein. As growth of this strain on high iron medium was improved it cannot be excluded that $PerR_{Cac}$ might also reveal some affinity to the *B. subtilis* Fur box. In *B. subtilis* the binding sites of Fur and PerR are highly similar, but so far examples for crossregulation by the two proteins have not been demonstrated (2, 21). Nevertheless, these data demonstrate that PerR of *C. acetobutylicum* acts as a hydrogen peroxide sensor *in vivo*. Previous observations have estimated that upon aeration the intracellular H_2O_2 concentration experienced by strict anaerobes could be substantially higher than in aerobes due to the oxidation of low potential flavoenzymes (30). Especially for a strict anaerobe it seems beneficial that the presence of O_2 is indirectly perceived by elevated levels of H_2O_2. Using H_2O_2 as a signal molecule also allows the integration of metabolic activity which determines the extent of ROS production. Consequently, *C. acetobutylicum* does not solely rely on their removal, but also attempts to adapt its fermentation metabolism to lesser reduced environments (Fig. 6). However, a broad understanding of its physiology during O_2 exposure will require further investigations, especially in respect to the biotechnological potential of this organism.

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4 DISKUSSION

4.1 Die transkriptionelle Regulation oxidativer Stressgene in Anaeroben

Die Existenz eines alternativen Detoxifikationssystem für O_2 und ROS in anaeroben Mikroorganismen (JENNEY *et al.*, 1999) warf zunächst die Frage auf, welche Relevanz ein solches System *in vivo* hätte. Ein erster Ansatzpunkt hierzu war die Untersuchung der Expression elementarer Bestandteile des Systems bei oxidativem Stress. Aerobe Bakterien besitzen verschiedene Mechanismen zur Wahrnehmung einer oxidativen Umgebung. Sie verwenden zumeist molekulare „Redox-Schalter", wie Fe-S-Cluster im SoxR/S System oder „Thiol-Schalter" wie in OxyR oder im Antisigmafaktor RsrA (POMPOSIELLO und DEMPLE, 2001; PAGET *et al.*, 1998; IMLAY, 2008b). Die Regulation der O_2-abhängigen Genexpression in Anaeroben war bisher kaum untersucht worden. Die zuvor als Peroxidasen beschriebenen normalen Rubrerythrine zeigten in den zuerst untersuchten Organismen wie *Porphyromonas gingivalis, C. perfringens* und *Campylobacter jejuni* nach oxidativem Stress keine oder nur eine sehr gering erhöhte Expression (SZTUKOWSKA *et al.*, 2002; JEAN *et al.*, 2004; YAMASAKI *et al.*, 2004). In Übereinstimmung mit diesen Ergebnissen war auch die Transkription der beiden normalen Rubrerythrin-Gene *rbr1* (*cac2575*) und *rbr2* (*cac3018*) in *C. acetobutylicum* kaum beeinflusst (MAY *et al.* 2004; HILLMANN, 2005). Dagegen war die Expression der reversen Rubrerythrin-Gene *rbr3A* (*cac3598*) und *rbr3B* (*cac3597*) sowohl auf Ebene der mRNA als auch des Proteins unter gleichen Bedingungen sehr stark induziert (MAY *et al.*, 2004; HILLMANN, 2005). Zeitgleich wurden in *C. acetobutylicum* noch weitere Gene identifiziert, die in Gegenwart von O_2 verstärkt exprimiert wurden (KAWASAKI *et al.*, 2005). Diese wurden mittlerweile größtenteils als funktionelle Bestandteile des reduktiven Detoxifikationssystems charakterisiert (RIEBE *et al.*, 2009, **Abb. 3**). Eine gemeinsame Regulation dieser Komponenten schien daher nicht unwahrscheinlich. Hierzu wurde innerhalb dieser Arbeiten zunächst eine genauere Transkriptionsanalyse des *rbr3* Operons vorgenommen und ein putativer Promotor postuliert. Darüber hinaus war sie die Grundlage zur Identifizierung PerRs als zentralem Repressor reverser Rubrerythrine und des gesamten alternativen Detoxifikationssytems.

4.1.1 Die PerR-abhängige Expression reverser Rubrerythrine und anderer oxidativer Stressproteine

Die 5'UTR des *rbr3* Operons enthielt eine AT-reiche, palindromische Sequenz (IR1), die in Teilen Ähnlichkeiten zur Consensussequenz des bereits länger bekannten „peroxide response regulator" PerR hat: In aeroben, Gram-positiven Bakterien werden verschiedene oxidative Stressgene, wie das der Katalase (*catA*) und der Alkylhydroperoxidase (*ahpC*) durch PerR kontrolliert (BSAT *et al.*, 1998; KING *et al.*, 2000; HORSBURGH *et al.*, 2001). PerR gehört zu einer Familie von Metalloregulatoren deren wichtigste weitere Mitglieder Fur- (ferric uptake regulator) und Zur- (zinc uptake regulator) ähnliche Proteine sind (LEE und HELMANN, 2007). Das Protein besitzt sowohl ein strukturell wichtiges Cys_4:Zn^{2+}-, als auch das Peroxid-empfindliche His_3Asp_2:Fe^{2+}-Zentrum (LEE und HELMANN, 2006a). In *B. subtilis* bindet PerR als Dimer an einen 16 Bp langes, AT-reiches Palindrom in der Promotorsequenz und reprimiert dadurch die Transkription nachfolgender Gene (MONGKOLSUK und HELMANN, 2002). Erst bei Peroxidstress kommt es zur Inaktivierung des Repressors. Der molekulare Vorgang dieser Inaktivierung wurde erst kürzlich genauestens beschrieben. Dabei kommt es in Gegenwart von H_2O_2 zu einer Eisen-katalysierten Oxidation eines Histidinrests im aktiven Zentrum, wodurch das Eisenzentrum zerfällt und die Bindung an die Zielsequenz verhindert wird (LEE und HELMANN, 2006a; TRAORÉ *et al.*, 2009).

Kürzlich durchgeführte *in silico* Analysen verwiesen bereits auf die Konserviertheit PerR ähnlicher Proteine in anaeroben Bakterien, dabei auch in einigen Gram-negativen (RODIONOV *et al.*, 2004). Innerhalb dieser Arbeit konnten drei Fur-ähnliche Proteine im sequenzierten Genom von *C. acetobutylicum* identifiziert werden. Allerdings wies nur eines davon (CAC2634) die konservierten Aminosäuren zum Aufbau eines Peroxid-empfindlichen Eisenzentrums auf. Das kodierende Gen wurde anschließend mittels einer auf einem „double crossover" basierenden homologen Rekombination durch eine Erythromycin-Resistenzkassette ersetzt (SOUCAILLE *et al.*, 2006). Erwartet wurde, dass die Abwesenheit des Repressors zu einer verstärkten, möglicherweise sogar konstitutiven Expression potentieller Zielgene führen würde. In *B. subtilis* und *Staphylococcus aureus* führte die Inaktivierung des *perR*-Gens tatsächlich zu einer Induktion des PerR-Regulons (BSAT *et al.*, 1998; KING *et al.*, 2000).
Auch in *C. acetobutylicum* kam es in Abwesenheit des *perR*-Gens zu einer massiv erhöhten Expression oxidativer Stressgene. Am stärksten waren davon das *rbr3*-

Operon, sowie ein bis zu diesem Zeitpunkt funktionell unbekanntes Flavoprotein (FprA1, CAC1027) betroffen. Letzteres war ebenfalls erstmalig in Gegenwart von O_2 aufgefallen (KAWASAKI *et al.*, 2004). Und auch hier konnte eine IR1 nahezu identische Sequenz 180 Nukleotide stromaufwärts des Startcodons nachgewiesen werden.
Interessanterweise ergab eine genomweite Suche nach IR1, dass dieses Palindrom nahezu vollständig vor allen Genen konserviert ist, die die höchsten transkriptionellen Induktionen in der *perR*-Mutante und O_2-gestressten Zellen des Wildtyps zeigten. Vor dem PerR-kodierenden Gen (*cac2634*) wurde ebenfalls eine IR1 Sequenz gefunden, hier kam es jedoch nur zu einer moderaten Expressionserhöhung. Dennoch kann von einer Autoregulation ausgegangen werden, wie sie bereits für homologe Gene aus *S. aureus* und *B. subtilis* gezeigt wurde (HORSBURGH *et al.*, 2001; FUANGTHONG *et al.*, 2002). In verwandten pathogenen Clostridien wie *C. perfringens* und *C. tetani* wurden ebenfalls ähnliche Sequenzen vor verschiedenen oxidativen Stressgenen identifiziert (**Tab. 2**).

Tab. 2: Putative PerR Boxen vor oxidativen Stressgenen in Clostridien[1]

Gen/ORF	Annotierte Funktion[2]	Sequenzmotiv und Abstand zum Startcodon
C. acetobutylicum		
Rbr3A	Reverses Rubrerythrin	AATaatTATTAtaTAATAacaATT (-109)
C. perfringens		
cpe0689	Reverses Rubrerythrin	tATtaaTATTAtaTAATAataATT (-123)
cpe0781	Flavoprotein	AATaatTATTAttTAATAaaaAgT (-127)
cpe0782	Alkylhydroperoxidase	AATaatTATTAttTAATAaaaAgT (-60)
C. tetani		
ctc00797	Thiolperoxidase	AATaatTATTAgcTAATAtaaATT (-22)
ctc00826	Reverses Rubrerythrin	AATtgtTATTAtaTAATAattATT (-35)
ctc01181	Arsenatreduktase	ATTtatTATTAatTAATActtATT (-17)
C. botulinum		
clb_1352	Reverses Rubrerythrin	AATgatTATTAttTAATAataAgT (-124)
clb_3250	Reverses Rubrerythrin	AATtacTATTAtaTAATAataATT (-62)

[1] Sequenzmotive wurden mit Hilfe der „virtual foot print" Software der PRODORIC Datenbank identifiziert (http://prodoric.tu-bs.de/vfp; MÜNCH *et al.*, 2003). Als zu suchendes Motiv wurde die Nukleotidsequenz 5'-AATNNNTATTANNTAATANNNATT-3' verwendet, mit „N" als unspezifischem Nukleotid.

[2] entsprechend der Datenbank des National Center of Biological Investigation, NCBI (http://www.ncbi.nlm.nih.gov)

Ein Sequenzvergleich aller IR1 ähnlichen Sequenzen in *C. acetobutylicum* ermöglichte die Modellierung einer „position weight matrix“ (CROOKS *et al.*, 2004). Diese könnte als putative PerR-Consensus-Bindesequenz von *C. acetobutylicum* betrachtet werden. Hierzu war jedoch zunächst der Nachweis notwendig, dass in *C. acetobutylicum* tatsächlich ein direkter Einfluss von PerR auf die Genexpression bestand. Dies konnte experimentell bestätigt werden: PerR aus *C. acetobutylicum* wurde als *Strep*-tag Fusionsprotein heterolog in *E. coli* überexprimiert und gereinigt. In darauf folgenden „Gelshift assays“ mit einem DIG-markierten DNA-Fragment der 5'UTR des *rbr3* Operons kam es zu einem verlangsamten elektrophoretischen Laufverhalten, wenn unterschiedliche Konzentrationen des PerR-Proteins zum Ansatz gegeben wurden. Diese Ergebnisse belegten, dass ein entscheidender Teil des O_2-Stimulons von *C. acetobutylicum*, einschließlich aller bisher bekannten Komponenten des alternativen Detoxifikationssystems, einer direkten negativen Kontrolle durch PerR unterliegt.

4.1.2 PerR als indirekter O_2-Sensor

In den fakultativ aeroben Bakterien *B. subtilis* und *S. aureus* sind Gene des PerR-Regulons, wie das der Katalase (*catA*) oder der Alkylhydroperoxidase (*ahpC*) bei aerobem Wachstum reprimiert und werden erst in Gegenwart äußeren H_2O_2 verstärkt transkribiert. Die regulatorische Beteiligung PerRs an ihrer H_2O_2-abhängigen Induktion ist seit längerem bekannt (BSAT *et al.*, 1998). Die Funktion PerRs als Sensor für H_2O_2 sowie die Kristallstruktur des Proteins wurden erst kürzlich detailliert beschrieben (LEE und HELMANN, 2006a; TRAORÉ *et al.*, 2009). Die regulatorische Beteiligung eines homologen Proteins an der O_2-abhängigen Genexpression in obligaten Anaerobiern warf demnach die Frage auf, wie diese Zellen die Anwesenheit des O_2 sensorisch wahrnehmen. Aufgrund der verhältnismäßig hohen Ähnlichkeit in der Aminosäuresequenz zum bereits charakterisierten PerR aus *B. subtilis* (61%), sowie der Existenz aller notwendigen Aminosäuren zur Ausbildung des Zn^{2+}, bzw. des Fe^{2+}-Zentrums, schien es wahrscheinlich, dass auch der Mechanismus der Eisen-katalysierten Histidinoxidation beim Protein aus *C. acetobutylicum* konserviert ist. Diese Vermutung konnte durch Komplementationsexperimente bestätigt werden. In *B. subtilis perR*-Defektmutanten kam es zu einer Überexpression der Katalase und die Zellen waren phänotypisch durch eine Hyperresistenz gegenüber H_2O_2 charkaterisiert (BSAT *et al.*, 1998). Wurde jedoch eine Kopie des *perR*-Gens aus *C. acetobutylicum* ins Genom dieser Mutante integriert, so wurde dieser hyperresistente Phänotyp mehr

als revertiert, denn die Sensibilität gegenüber H_2O_2 überstieg die des Wildtyps. Demzufolge lag auch die Aktivität der Katalase in komplementierten Zellen bei aeroben Wachstumsbedingungen unter dem Niveau des Wildtyps. Externe H_2O_2-Konzentrationen führten jedoch erneut zu einer Aktivitätssteigerung. Diese Daten beseitigten mögliche Zweifel an der funktionellen Konserviertheit des PerR-Proteins aus *C. acetobutlicum* und verwiesen auf H_2O_2 als eine Art „second messenger" der O_2-Sensorik. Ähnliches wurde bereits zuvor für den Induktionsweg des OxyR-Regulons des verhältnismäßig aerotoleranten *Bacteroides fragilis* vermutet (ROCHA und SMITH, 1999).

Speziell obligate Anaerobier begegnen wahrscheinlich in Gegenwart von O_2 ungewöhnlich hohen intrazelluären H_2O_2-Konzentrationen (IMLAY, 2003 und 2008). Dies ist insbesondere auf deren weitreichende Verwendung reduzierter Flavine als Cofaktoren vieler Dehydrogenasen und Dehydratasen zurückzuführen. Diese Flavoenzyme autoxidieren in Gegenwart von O_2 und produzieren ein Gemisch aus O_2^- und H_2O_2 (IMLAY, 2003). Die Geschwindigkeit der Reaktion mit O_2 ist dabei sehr von der Position des Cofaktors im oder am Protein abhängig. Je exponierter die Lage, desto höher ist die Rate der Autooxidation und damit auch die Produktion reaktiver O_2-Spezies (MASSEY, 1994; MESSNER und IMLAY, 2002; IMLAY, 2003). Flavoenzyme mit FAD als Cofaktor sind auch im Stoffwechsel von Clostridien von zentraler Bedeutung, so z. B. bei der Butyryl-CoA Dehydrogenase, den Dehydrogenase assozierten ETFs (Elektronentransfer-Flavoproteine) und der Hydroxybutyryl-CoA-Dehydratase (BENNET und RUDOLPH, 1995; BOYNTON *et al.*, 1996; SCHERF und BUCKEL, 1993). Es ist deshalb wahrscheinlich, dass eine hohe Aktivität dieser Enzyme in Gegenwart von O_2 auch zu einer drastisch erhöhten Produktion von H_2O_2 führt. Erst die Bildung von H_2O_2 würde dann zur Induktion des PerR Regulons führen. Es stellt sich die Frage warum H_2O_2 als „second messenger" verwendet wird, denn Proteine, die als direkte O_2 Sensoren wirken (PAS Domänen, FNR) sind seit längerem bekannt und in allen Reichen des Lebens verbreitet (ZHULIN *et al.*, 1997; JORDAN *et al.*, 1997). Die Verwendung von PerR und H_2O_2 brächte allerdings den zusätzlichen Vorteil, dass neben der bloßen Gegenwart des O_2 gleichzeitig metabolische Signale integriert werden könnten. Denn letztendlich ist die Produktion von H_2O_2 und O_2^- eine Kombination aus O_2-Konzentration und metabolischer Aktivität. Bei geringer Aktivität wäre demzufolge auch eine abgeschwächte Induktion des PerR-Regulons ausreichend, um Schäden zu vermeiden.

Darüber hinaus ist die H_2O_2-vermittelte Histidinoxidation bei PerR nach neueren Erkenntnissen auch von der Zusammensetzung und Konzentration intrazellulärer

Metalle abhängig (LEE und HELMANN, 2007). So konnten je nach Zusammensetzung des Mediums mindestens zwei unterschiedliche Metallierungszustände PerRs gefunden werden, die auch in ihrer Oxidationsempfindlichkeit zwischen hoch (Fe^{2+}) und niedrig (Zn^{2+}) variierten (LEE and HELMANN, 2006b). Freies oder Nukleotid-komplexiertes, intrazelluläres Fe^{2+} bestimmt als Katalysator der Fentonreaktion maßgeblich die Bildung des Hydroxyl-Radikals (HO•), das mit diffusionslimitierten Raten mit Proteinen und vor allem mit Nukleinsäuren reagiert (WALLING, 1975; IMLAY und LINN, 1988; DEAN *et al.*, 1997; IMLAY, 2003; **Abb. 6**).

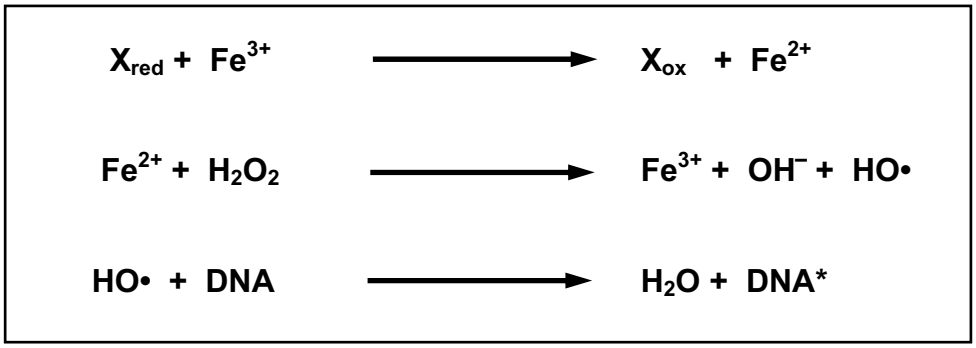

Abb. 6: Die Fentonreaktion *in vivo* (IMLAY, 2003; mod.)

X, unspezifische Elektronendonatoren z. B. NAD(P)H, $FADH_2$, Cystein und Glutathion; **red**, reduziert; **ox**, oxidiert; **DNA***, unspezifische DNA Schäden

Die zentrale Rolle von Fe^{2+} für das Überleben in oxidativer Umgebung konnte auch während dieser Arbeit bestätigt werden. Die Funktion divalenter Kationen bei der Regulation durch PerR in *C. acetobutylicum* wurde hingegen bisher nicht untersucht. Dennoch legen sowohl die strukturelle, als auch funktionelle Konserviertheit nahe, dass bei PerR aus *B. subtilis* und *C. acetobutylicum* ähnliche Affinitäten zu divalenten Kationen auftreten. Insgesamt beschränkt sich die Rolle PerRs daher wohl nicht nur auf eine indirekte Messung intrazellulären O_2, sondern das Protein könnte eher als „Frühwarnsystem“ und komplexer Sensor für verschiedene Signale wirken, die in letzter Konsequenz zu oxidativen Schäden führen. Dies sind nach derzeitigem Kenntnisstand vor allem die eigene metabolische Aktivität und die intrazelluläre Eisenkonzentration. Unter diesen Gesichtspunkten scheint es verständlich, dass gerade ein solches, hochentwickeltes Sensor/Regulator-System einem obligat anaeroben Bakterium eine Adaptation an kontinuierliche O_2-Zufuhr ermöglicht.

4.1.3 PerR als Transkriptionsregulator

Die bisherigen Erkenntnisse zur PerR-Regulation zeichnen fast vollständig das Bild eines „klassischen“ Transkriptionsrepressors. Eine direkte Bindung an ein 19 Bp langes, konserviertes AT-reiches Palindrom wurde bereits für homologe Proteine aus *B. subtilis*, *S. aureus* oder *S. pyogenes* nachgewiesen (BSAT *et al.*, 1998; KING *et al.*, 2000; HORSBURGH *et al.*, 2001). Zusätzlich zeigten globale Expressionsanalysen, dass die Abwesenheit dieses Regulators zu einer starken Aktivierung nahezu aller Zielgene führt (HELMANN *et al.*, 2003; LI *et al.*, 2004; BRENOT *et al.*, 2007). Die Bindestellen von PerR liegen zumeist in unmittelbarer Nähe der -35- und -10-Region oder überlappen diese (HERBIG und HELMANN, 2001; BRENOT *et al.*, 2005). Beim *rbr3*-Operon von *C. acetobutylicum* befindet sich die PerR-Box jedoch eher zwischen dem ermittelten Transkriptionsstartpunkt (168 Bp stromaufwärts) und dem Startcodon von Rbr3A (102 bp stromabwärts). Dieser Abstand erscheint zunächst unvereinbar mit dem derzeitigen Modell. Einen Ansatz zur Klärung dieses Widerspruchs liefert möglicherweise der nahe verwandte und am besten untersuchte „ferric uptake repressor“ Fur. Hier wurde häufig, ausgehend von der Fur-Box, eine Polymerisation von Dimeren entlang der DNA beobachtet (BAICHOO und HELMANN, 2002). Diese helikalen Strukturen von Fur-Polymeren waren sogar mikroskopisch sichtbar (LeCAM *et al.*, 1994). Sie entstehen nicht zufällig durch einen Überschuss an Regulatorprotein sondern sind das Ergebnis wenig konservierter, hexamerer DNA-Sequenzen in der Umgebung, die zur Entstehung eines „ausgeweiteten Operators“ führen (ESCOLAR *et al.*, 2000). Die AT-reiche DNA von *C. acetobutylicum*, sowie die Existenz weiterer IR1 ähnlicher Sequenzen in der Promotorregion, widersprechen nicht der Existenz eines ähnlichen Mechanismus zur Repression der *rbr3*-Gene. Letztendlich ist aber auch in anderen Organismen noch nicht näher untersucht worden, wie genau DNA gebundene PerR-Moleküle zur Transkriptionsrepression führen. Es wird daher angenommen, dass die Bindung von PerR sterisch entweder die Bildung eines „open complex“, oder die Bindung der RNA Polymerase an die Promotorsequenz verhindert (LEE und HELMANN, 2007).

Selbst die Rolle PerRs als reiner Repressor wurde in den letzten Jahren widerlegt. So wirkt der sehr sequenzhomologe *„Borrelia* oxidative stress response regulator“ BosR (50 % Aminosäure-identität zu PerR aus *B. subtilis*) als Transkriptionsaktivator der in Gegenwart von Peroxiden weit stromaufwärts des Promotors bindet und so die Expression des Eisen-bindenden Dps Protein induziert (BOYLAN *et al.*, 2003). Auch für *B. subtilis* und *S. pyogenes* wurde eine direkte positive Regulation des *srfA*-

(surfactin A) Operons bzw. des *csp*- (cold shock protein) Gens durch DNA-Bindung von PerR gezeigt (HAYASHI *et al.*, 2005; BRENOT *et al.*, 2005). Eine Aktivatorfunktion PerRs kann auch für *C. acetobutylicum* postuliert werden (**Abb. 7**): Zum einen war die Anzahl der Gene, die in Abwesenheit von PerR mehr als dreifach schwächer transkribiert wurden unerwartet groß (93 Gene). Sie überstieg sogar die Anzahl der dreifach stärker exprimierten Gene (51 Gene). Dies könnte auf einen indirekten Effekt des Fehlens von PerR, nämlich die Derepression eines oder mehrerer anderer Repressoren, zurückzuführen sein. Diese sollten dann aber auch unter O_2 (Inaktivierung PerRs) tendenziell schwächer exprimiert werden. Es konnten allerdings keine Gene identifiziert werden, die sowohl unter O_2 im Wildtyp, als auch unter anaeroben Bedingungen in der *perR*-Mutante drastisch schwächer exprimiert wurden.

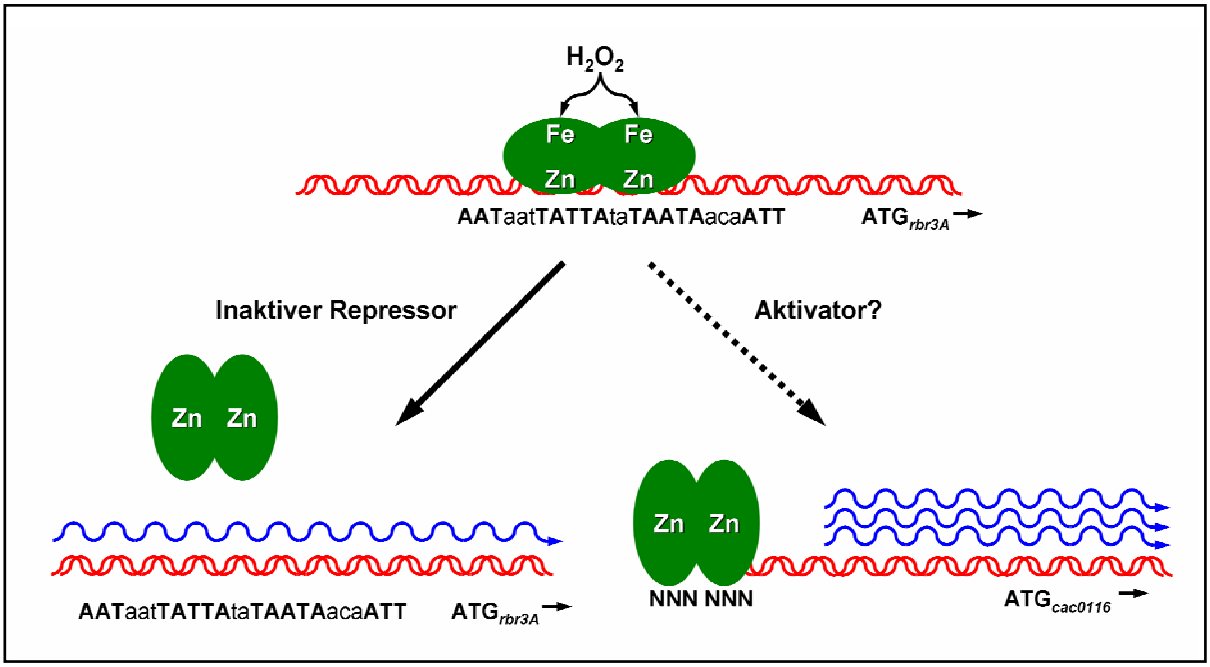

Abb. 7: Modell zur regulatorischen Funktion von PerR in *C. acetobutylicum*

Am PerR Dimer (**grün**) werden die Fe-Zentren durch H_2O_2 inaktiviert (HELMANN *et al.*, 2006a), wodurch sich die Konformation des Dimers ändert und PerR sich von der Bindestelle der DNA (**rot**) ablöst. Im Fall des *rbr3* Operons kommt es dann zur Synthese der mRNA (**blau**). Alternativ könnte eine Aktivierung der Transkription von *cac0116* durch Bindung des inaktiven Repressors an eine unbekannte Nukleotidsequenz (NNN NNN) im Promotorbereich erfolgen.

Im Fall des Gens einer unbekannten Kohlenmonoxid-Dehydrogenase (*cac0116*) ergibt sich sogar ein völlig umgekehrtes Bild: in Gegenwart von O_2 war es in Zellen des Wildtyps eines der am stärksten transkribierten Gene. Mit einer fast 30fachen Induktion erreichte die Transkriptmenge etwa 50 % des Wertes für *rbr3.* Interessanterweise war *cac0116* dagegen schon unter anaeroben Wachstumsbedingungen in der *perR*-Deletionsmutante etwa 30-fach weniger exprimiert als in Zellen mit dem Wildtypallel und ist damit an der unteren Nachweisgrenze des Microarrays. Selbst wenn beide Stämme unter O_2-Zufuhr wuchsen, blieb die Expression in den Zellen des Wildtyps um mehr als das 10-fache stärker (unveröffentlichte Daten). Die naheliegendste Erklärung ist, dass ein für die Expression sehr entscheidender Faktor fehlt. Ob es sich dabei um PerR selbst handelt, kann anhand der vorliegenden Ergebnisse nicht bewiesen werden. Dennoch ist es sehr wohl denkbar, dass selbst die oxidierte, und als Repressor inaktive, Form PerRs nicht gänzlich ohne regulatorische Funktion ist (**Abb. 7**). Möglicherweise erkennt dieses Protein andere Zielsequenzen und wirkt dort als Aktivator, ähnlich wie BosR bei *napA* oder PerR am *srfA*-Operon. Auch eine indirekte Aktivierung von Genen des Eisenstoffwechsels über kleine RNAs (sRNA) ist bereits mehrfach bei den nahe verwandten Fur-Proteinen nachgewiesen worden (MASSE und GOTTESMANN, 2002; WILDERMANN *et al.*, 2004).

An dieser Stelle sei auch erneut auf die potentielle Bindung und regulatorische Rolle anderer divalenter Kationen verwiesen (vgl. 4.1.2). Eine Beteiligung des hier untersuchten PerR-Proteins an der Regulation des Metallstoffwechsels oder des Transports ist allerdings unwahrscheinlich. Denn durch ICP-Analysen („inductively coupled plasma“) ließ sich feststellen, dass die Deletion des *perR*-Gens kaum Auswirkung auf das intrazelluläre „Metallom“ des Bakteriums hat. Auch die O_2-abhängige Expression von Fe^{2+}-Transporter-Operonen, wie aus den Transkriptionsdaten ersichtlich, geschah weitestgehend unabhängig von PerR. Es soll jedoch an dieser Stelle nicht unerwähnt bleiben, dass sich im Genom von *C. acetobutylicum* noch ORFs für zwei weitere, bisher nicht charakterisierte Fur-Proteine, CAC1682 (Fur?) und CAC0931 (Zur?) befinden. So kann abschließend festgestellt werden, dass die hier gewonnen Erkenntnisse zwar einen ersten Einblick in die PerR vermittelte Genregulation anerober Bakterien gewähren, gleichzeitig aber auch die einzigartige regulatorische Komplexität dieser Familie von Metalloregulatoren erkennen lassen.

4.1.4 Die PerR-unabhängige Regulation oxidativer Stressgene

Die innerhalb dieser Arbeit erzielten Ergebnisse zur Regulation des *rbr3*-operons und anderer oxidativer Stressgene lassen die Schlussfolgerung zu, dass außer PerR bereits auf Transkriptionsebene noch weitere Faktoren an der Genexpression beteiligt sind. Sowohl die Transkriptomdaten dieser Arbeit, als auch gezielte Expressionsanalysen (KAWASAKI *et al.*, 2005) lassen klar erkennen, dass *C. acetobutylicum* in Gegenwart von O_2 offensichtlich ein weiteres System zur Detoxifizierung und Balancierung des Redoxgleichgewichts verwendet. Dieses könnte im Gegensatz zum erst kürzlich entdeckten NADH- und Rubredoxin abhängigen System, weitestgehend auf NADPH und Glutaredoxin/Thioredoxin basieren. Letztere sind aus fast allen aeroben Organismen bekannt und sind neben der Abwehr oxidativen Stresses auch an der Synthese von (Desoxy)Ribonukleotiden, der Bereitstellung reduzierten Schwefels, der Signaltransduktion und der Thiolreduktion von Proteinen beteiligt (FERNANDES und HOLMGREN, 2004).

In *C. acetobutylicum* war die Transkription fast aller Gene, die für Thioredoxine (*cac0869, cac1547*) und die entsprechende Reduktase (*cac1548*), sowie für mehrere Glutathionperoxidasen (*cac1549, cac1570, cac1571*) kodieren, in den Zellen des Wildtyps unter O_2 induziert (KAWASAKI *et al.*, 2005). Die Deletion *perR*s blieb aber ohne signifikanten Einfluß auf deren Expression. Ein ähnliches Bild einer PerR-unabhängigen, aber dennoch O_2-abhängigen Expression zeigte sich auch für Gene für deren Proteinprodukte Funktionen bei der DNA-Reparatur, der Aufnahme von Fe^{2+}-Ionen, und des Butyratweges angenommen werden können. Es ist daher sehr wahrscheinlich, dass in *C. acetobutylicum* unter O_2 neben PerR weitere, bisher allerdings noch nicht identifizierte Sensor/Regulatorsysteme existieren.

In bereits eingehender untersuchten Bakterien wirken ebenfalls mehrere, unabhängige oxidative Stressregulons. In *E. coli* kontrollieren die Redoxschalter SoxRS und OxyR u. a. die Expression von *sodA* (Superoxid Dismutase), *micf* (regulatorische RNA), *katG* (Katalase), *ahpCF* (Alkylhydroperoxidase) und *grxA* (Glutaredoxin) als Transkriptionsfaktoren (ZHENG *et al.*, 1999; POMPOSIELLO und DEMPLE, 2001). Auch der *C. acetobutylicum* phylogenetisch näher stehende *B. subtilis* nutzt neben PerR noch OhrR, einen Sensor für organische Hydroperoxide, der durch diese inaktiviert wird (FUANGTHONG und HELMANN, 2002). FNR (Fumarat- und Nitratreduktaseregulator) ist ein weiterer Redoxschalter der sowohl in *E. coli* als auch in *B. subtilis,* in Abwesenheit von O_2, Gene der anaeroben Atmung aktiviert (SHAW *et al.*, 1983; KILEY und REZNIKOFF, 1991; GREEN *et al.*, 1996; REENTS *et al.*, 2006).

Insgesamt scheinen aber diese typischen Sensorsysteme wie SoxRS, OxyR und FNR in *C. acetobutylicum* weniger konserviert zu sein. Kein Gen, das diese Regulatoren kodiert, konnte bisher zweifelsfrei im Genom von *C. acetobutylicum* identifiziert werden (NÖLLING *et al.*, 2001). Auch in BLAST-Analysen des Genoms konnten lediglich offene Leserahmen mit geringen Ähnlichkeiten zu bereits beschriebenen Redox-Regulatoren identifiziert werden (**Tab. 3**).

Tab. 3. ORFs aus *C. acetobutylicum* mit Ähnlichkeiten zu SoxR, SoxS, Oxyr, FNR und OhrR[1]

Protein	Acc. Nr. / ORF	Aminosäuresequenz Identität/ Ähnlichkeit (%)	Score (BLOSUM 40)
SoxR *E. coli*	AAB31680	100 / 100	1166
	Cap0178	27 / 47	202
	Cac2451*	26 / 43	177
SoxS *E. coli*	P0A9E2	100 / 100	822
	Cac0426*	34 / 67	256
OxyR *E. coli*	AAF06745	100 / 100	2183
	Cac3361	24 / 48	444
FNR *B. subtilis*	P46908	100 / 100	1728
	Cac1511	26 / 57	342
OhrR *B. subtilis*	O34777	100 / 100	1049
	Cac1569	51 / 75	542

[1] Die ORFs aus *C. acetobutylicum* wurden zunächst durch genomische „Blast"-Analysen mit den Proteinen aus *E. coli* und *B. subtilis* identifiziert (CUMMINGS *et al.*, 2002) und anschließend mit der „EMBOSS align" Software verglichen (RICE *et al.*, 2000).

* Nur die N-terminale Domäne des ORFs aus *C. acetobutylicum* wurde verglichen.

Allerdings könnte ein putatives OhrR-Protein von Interesse sein, das durch den ORF *cac1569* kodiert wird. Dieses weist nicht nur die höchste Ähnlichkeit zu bereits beschriebenen Regulatoren auf, sondern enthält auch das konservierte Cystein am N-Terminus, das in *B. subtilis* als Sensor für organische Peroxide dient (FUANGTHONG und HELMANN, 2002; PANMANEE et al., 2006; **Abb. 8A**). Weiterhin ist *cac1569* genomisch in unmittelbarer Nähe der unter O_2 PerR-unabhängig exprimierten Gluthathionperoxidasen BsaA2 und BsaA3 (CAC1570/1) lokalisiert. Hier wäre zum Nachweis eine experimentelle Untersuchung sicherlich vielversprechend. Im Fall eines SoxR-ähnlichen Proteins (CAC2451) ist dies bereits unternommen worden.

Interessanterweise ist das Gen Teil des von KAWASAKI *et al.* (2005) beschrieben O_2-abhängigen Genclusters, dass innerhalb dieser Arbeit als Teil des PerR Regulons identifiziert wurde. Bisher konnte eine regulatorische Funktion des Proteins aber nicht nachgewiesen werden (SCHEEL, 2008).

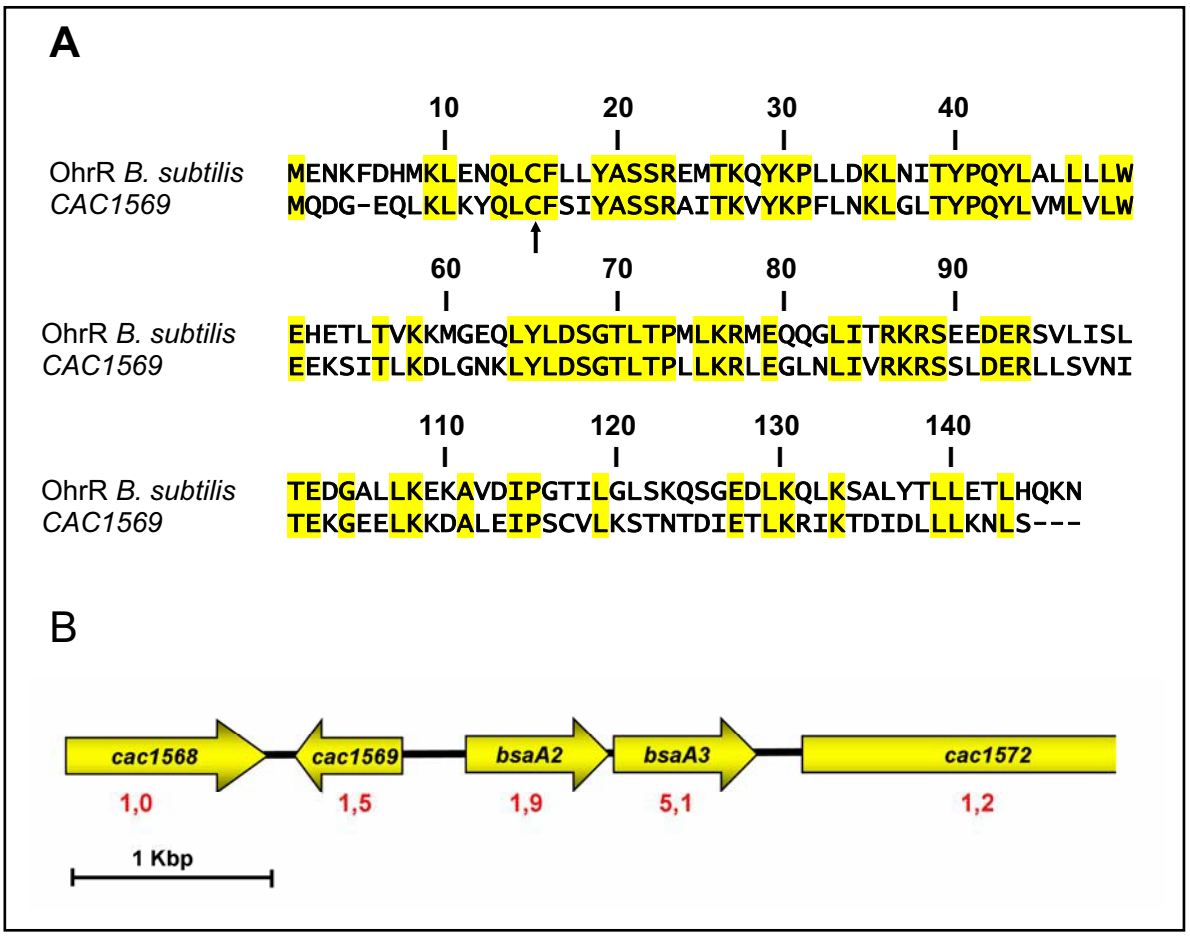

Abb. 8: CAC1569 - ein potentieller Regulator bei oxidativem Stress in *C. acetobutylicum*

A: Vergleich der Aminosäuresequenzen des Response Regulators für organische Hydroperoxide aus *B. subtilis* (**OhrR**, FUANGTHONG und HELMANN, 2002) und des ORFs **CAC1569** aus *C. acetobutylicum*. Zahlen oberhalb der Sequenz kennzeichnen die Position der Aminosäuren. Übereinstimmende Sequenzbereiche sind gelb hinterlegt. Der Pfeil markiert das konservierte, sensorische Cystein an Position 15.

B: Die genomische Umgebung von ***cac1569***. Induktionsfaktoren der Gene unter O_2 sind rot dargestellt. ***cac1568*****,** putative Diguanylat Cyclase/Phosphodiesterase; ***bsaA2*** und ***bsaA3***; Gluthathionperoxidasen; ***cac1572***, putative Fruktose-1,6-bisphosphatase

Die bisherigen Ergebnisse zur Transkription des *rbr3* Operons schließen ebenfalls nicht aus, dass in *C. acetobutylicum* ein oder mehrere alternative Sigmafaktoren an der Expression oxidativer Stressgene beteiligt sind. Hierfür spricht insbesondere, dass in

der -35- und -10-Region (TTATCA bzw. AACTTT) deutliche Abweichungen vom Consensuspromotor des vegetativen σ^{70}-Faktor Gram-positiver Bakterien gefunden wurden (TTGACA-TATAAT, GRAVES und RABINOWITZ, 1986). Die Beteiligung alternativer Sigmafaktoren an der Transkription oxidativer Stressgene ist u. a. aus den ebenfalls Gram-positiven *B. subtilis* (σ^B) und *Streptomyces coelicolor* (σ^R) bekannt (VÖLKER *et al.*, 1999; KANG *et al.*, 1999). Der Faktor σ^B kontrolliert in *B. subtilis* zahlreiche Gene mit erhöhter Expression in der stationären Wachstumsphase. Für die Existenz eines σ^B-homologen Proteins in Clostridien gibt es bisher allerdings keine Hinweise (HECKER und VÖLKER, 2001). Im Gegensatz dazu wurde erst kürzlich ein Operon (*cac3267-cac3265*) identifiziert, das für ein ECF-σ-Faktor-System (extracytoplasmic function) kodiert und dessen Proteine Sequenzähnlichkeiten zu σ^R und dem dazugehörigen, redoxregulierten und membrangebundenen Antisigmafaktor RsrA aufweisen (BARTHEL, 2008). Die Rolle dieses Systems in der oxidativen Stressantwort und der Einfluss auf die Expression des *rbr3* Operons werden derzeit untersucht. Ein Aspekt unterstützt die Theorie zur Beteiligung eines sensorischen, membrangebundenen Antisigmafaktors: Die transkriptionelle Induktion des Operons durch alternative, extrazelluläre Stressfaktoren (Lösungsmittel, erhöhter äußerer pH, NaCl) mag z. T. unspezifisch sein, ist aber jedenfalls für Ethanol auch bei der PerR-unabhängigen Regulation oxidativer Stressgene in *S. pyogenes* aufgefallen. Dort wurde ebenfalls die Beteiligung eines unbekannten σ-Faktors vermutet (KING *et al.*, 2000). Es wäre durchaus denkbar, dass solch ein zweites sensorisches System verwendet wird, das als eine Art „Frühwarnsystem“ bereits äußere Signale wahrnimmt. Hierzu wäre es z. B. interessant, ob eine O_2-unabhängige Veränderung des Redoxpotentials ebenfalls zu einer erhöhten Expression *rbr3*s führt. Zum Nachweis einer Beteiligung weiterer Regulatoren wäre die *perR*-Deletionsmutante zudem ein hervorragendes experimentelles System, da die Expression des Operons auch unabhängig von PerR analysiert werden könnte.

4.2 Anpassungen an mikroaerobe Wachstumsbedingungen

Frühere Arbeiten lieferten die Erkenntnis, dass *C. acetobutylicum* durchaus zu einem „mikroaeroben“ Wachstum mit O_2 befähigt ist, dieses aber sofort zum Erliegen kommt, wenn sich O_2 im Medium anreichert (O'BRIEN und MORRIS, 1971; KAWASAKI *et al.*, 2004). Die aerotolerante *perR*-Deletionsmutante von *C. acetobutylicum* zeigte demgegenüber jedoch ein abweichendes Verhalten: Eine dereprimierte Expression des PerR-Regulons führte zu einer drastisch erhöhten O_2-Toleranz, die anaerob vorinkubierten Kolonien anhaltendes Wachstum unter atmosphärischen O_2-Konzentrationen ermöglichte. In aerober Flüssigkultur ergab sich ein ähnliches Bild: Trotz der Gegenwart von gelösten O_2 kam es zu einem zeitlich limitierten Anstieg der optischen Dichte und der Lebendzellzahl (LZZ). Die LZZ der *perR*-Mutante blieb bis zu 6 Stunden lang über dem Wert bei Beginn der Belüftung, während die des Wildtyps bereits nach 30 min auf unter 0,1 % des Ausgangswertes sank. Im Verlauf der näheren Charakterisierung dieser Mutante konnte ein erhöhter O_2 Verbrauch der Zellen nachgewiesen werden, was die Vermutung nahe legte, dass die Zellen durch hohen kontinuierlichen O_2 Verbrauch Anaerobiose aufrechterhielten. Diese kann jedoch nur zum Teil für die erhöhte Aerotoleranz verantwortlich sein, da die *perR*-Mutante auch bei geringsten Zelldichten, starker Belüftung und in Gegenwart von gelöstem O_2 überlebte. Die Aktivität und Expression der SOD blieben währenddessen eher gering und ein Katalase kodierendes Gen konnte bisher nicht identifiziert werden (NÖLLING *et al.*, 2001). Eine Konsequenz war daher die Frage, welche alternativen Mechanismen entscheidend zur Aerotoleranz beitragen, bzw. welche Faktoren letztendlich limitierend sind. Einige Ergebnisse dieser Arbeit, die zur Klärung dieser Fragestellung beitragen, sollen im Folgenden diskutiert werden.

4.2.1 Koloniemorphologie

Zu den bereits früher beobachteten Eigenschaften von *C. acetobutylicum* gehört die Fähigkeit, beim Wachstum auf Festmedium morphologisch komplexe Kolonien auszubilden. Die Form und Oberflächenstruktur dieser im Durchmesser ca. 1-5 mm großen Kolonien sind sehr variabel und abhängig von der Zusammensetzung des verwendeten Festmediums, dem Grad der Lösungmittelproduktion sowie der Sporulationsrate (ADLER und CROW, 1987; CLARK *et al.*, 1989). Strukturelle Analysen verwiesen zudem auf eine „proteinöse“ Hülle an der Peripherie der Kolonien

(JONES *et al.*, 1980). Im Zusammenhang mit dieser Arbeit war von besonderem Interesse, dass 2-3 Tage alte, anaerob gewachsene Kolonien bei weiterer aerober Inkubation unter atmosphärische O_2-Konzentrationen Fruchtkörper ähnliche Strukturen ausbildeten (JONES *et al.*, 1980; **Abb. 9**). Dabei zeigte sich innerhalb von 4-5 Tagen, ausgehend vom Zentrum der Kolonie, ein ca. 2-4 mm langer, schlauchförmiger Fortsatz. An dessen Spitze befanden sich primär vollständig ausgebildete Sporen (JONES *et al.*, 1980). Dies ist eine bisher sehr wenig erforschte Parallele zu den am besten untersuchten prokaryotischen Fruchtkörpern von *Myxococcus xanthus* (SHIMKETS, 1987; SHIMKETS, 1999; KAISER, 2003).

Abb. 9: Kolonien von *C. acetobutylicum* nach aerober Inkubation
Zellen von *C. acetobutylicum* wurden für 48 h bei 37 °C auf Komplexagar in einer N_2:H_2 (9:1) Umgebung kultiviert. Ausschließlich nach einer weiteren aeroben Inkubation für 100-150 h waren vereinzelt die bereits früher von ADLER *et al.* (1987) beobachteten Fruchtkörper ähnlichen Strukturen sichtbar.

Die O_2-abhängige Ausbildung dieser Strukturen wurde auch hier für viele Kolonien des Wildtyps beobachtet. Sie erfolgte jedoch keinesfalls gleichmäßig und war bei der Mehrheit der Kolonien nur in Ansätzen sichtbar, so dass bisher keine genaueren Angaben zu den Bedingungen der Induktion gemacht werden können. Es ist lediglich zu vermuten, dass hier der Kombination aus Zeitpunkt der O_2-Exposition und Alter der Kolonie eine entscheidende Rolle zukommt. Einheitlich war dagegen ein vollständiger Stillstand des Flächenwachstums. Die Deletion von *perR* änderte beide Eigenschaften dieses Phänotyps. Das Wachstum setzte sich über mehr als 10 Tage fort, ohne dass Fruchtkörper ausgebildet wurden. Dabei erreichten die Kolonien einen Durchmesser der selbst den von völlig anaerob gewachsener Kolonien des Wildtyps deutlich

übertraf. Worin diese phänotypische Veränderungen begründet liegen, ist ebenso unklar wie die Mechanismen, einer O_2-abhängigen Bildung von Fruchtkörpern. Bekannt war zu Beginn dieser Arbeit lediglich, dass die Bildung der Fruchtkörper an die Sporulation gekoppelt ist und beides durch O_2 induziert werden kann (JONES *et al.*, 1980; ROSS *et al.*, 1990). Möglicherweise liegen die Gründe für das Ausbleiben der Fruchtkörper indirekt in der hohen O_2/ROS-Reduktionsrate der *perR*-Mutante, z. B. durch Veränderung des intrazellulären Redoxgleichgewichts zwischen NADH und NAD^+ zugunsten des NAD^+. Es ist wäre in jedem Fall interessant zu erfahren, welche Mechanismen ein anaerobes Bodenbakterium befähigen, in Gegenwart atmosphärischer O_2-Konzentrationen (wie z. B. an der Erdoberfläche) eine komplexe Struktur auszubilden, die eine Verbreitung von Sporen fördern könnte.

4.2.2 Reduktive Detoxifizierung

Reduktive Mechanismen zur Detoxifizierung von O_2 und ROS sind in anaeroben Mikroorganismen wohl weiter verbreitet als ursprünglich angenommen. Gerade während der letzten Jahre konnten O_2-reduzierende 2-Fe-Flavoproteine, Superoxidreduktasen (O_2^-) und Rubrerythrine (H_2O_2) in den verschiedensten Anaeroben nachgewiesen werden. Dies schließt Archaeen, Gram-negative und Gram-positive Eubakterien und sogar eukaryotische Organismen ein (JENNEY *et al.*, 1999; LUMPPIO *et al.*, 2001; MAY *et al.*, 2004; PÜTZ *et al.*, 2005). Obwohl konkrete zeitliche und phylogenetische Angaben zur Evolution von Prokaryoten bisher schwierig sind (BATISTUZZI *et al.*, 2004), lässt sich dennoch schlussfolgern, dass Mechanismen zur Detoxifizierung von ROS und ihre Regulation bereits lange vor der Entwicklung aerober Stoffwechselwege existierten.

Die Effektivität der Reduktion von ROS und O_2 in Anaeroben zeigt sich insbesondere am Beispiel der *perR*-Deletionsmutante von *C. acetobutylicum*: Diese zeigte nicht nur massiv erhöhte Peroxidase- und NADH-Oxidaseaktivität im Proteinextrakt, sondern auch eine O_2-Verbrauchsrate, die mit ca. 8 fmol $\cdot$ h^{-1} $\cdot$Zelle^{-1} fast der atmender *E. coli*-Zellen entsprach (IMLAY, 2008a). Die vollständige Reduktion von O_2 ermöglicht *C. acetobutylicum* trotz kontinuierlicher Zufuhr eine „Quasi“-Anaerobiose aufrechtzuerhalten. Dies wurde bereits durch frühere Beobachtungen bestätigt: Obwohl exponentiell wachsenden Zellen kontinuierlich 5 % O_2 zugeführt wurde, lag die O_2-Konzentration im Wachstumsmedium im gesamten Zeitraum bei 0,0-0,1 % und die Wachstumsrate blieb nahezu unverändert (KAWASAKI *et al.*, 2004).

Erst kürzlich sind mit Desulfoferrodoxinen und Rubrerythrinen Enzyme zur Reduktion von O_2^- bzw. von H_2O_2 und O_2 funktionell charakterisiert worden (RIEBE *et al.*, 2007; RIEBE, 2009; RIEBE *et al.*, 2009). Die Präferenz der Rubrerythrine für H_2O_2 warf allerdings die Frage auf, ob diese tatsächlich allein für den hohen O_2-Verbrauch verantwortlich sind. Innerhalb dieser Arbeit wurden dazu die zwei 2-Fe-Flavoproteine („flavodiironproteins“ oder FDPs) FprA1 und FprA2 untersucht, die zu den wenigen FDPs gehören für die bereits früher eine O_2-abhängige Expression gezeigt werden konnte (KAWASAKI *et al.*, 2005). FDPs sind nicht nur in anaerob lebenden Prokaryoten hochkonserviert, sondern kommen auch in einigen mikroaeroben Protozoen vor (SARTI *et al.*, 2004; DI MATTEO *et al.*, 2008). Sie besitzen FMN als C-terminalen Cofaktor, sowie das charakteristische, schwefellose Fe-Fe Zentrum am N-Terminus (KURTZ, 2007). FDPs ist außerdem gemeinsam, dass ihre Reduktaseaktivität von intermediären Elektronenüberträgern wie Rubredoxin oder $F_{420}H_2$ (methanogene Archaeen) abhängt (FRAZAO *et al.*, 2000; SEEDORF *et al.*, 2004; KURTZ, 2007). Für die meisten der bereits charakterisierten Mitglieder wurden sowohl O_2, als auch NO als terminale Elektronenakzeptoren identifiziert (SILAGHI-DUMITRESCU *et al.*, 2003 und 2005; KURTZ, 2007).

Beide untersuchten Enzyme, FprA1 und FprA2, waren in der Lage Elektronen von reduziertem Rubredoxin entweder auf O_2, oder Stickstoffmonoxid (NO) zu übertragen (**Abb. 10**).

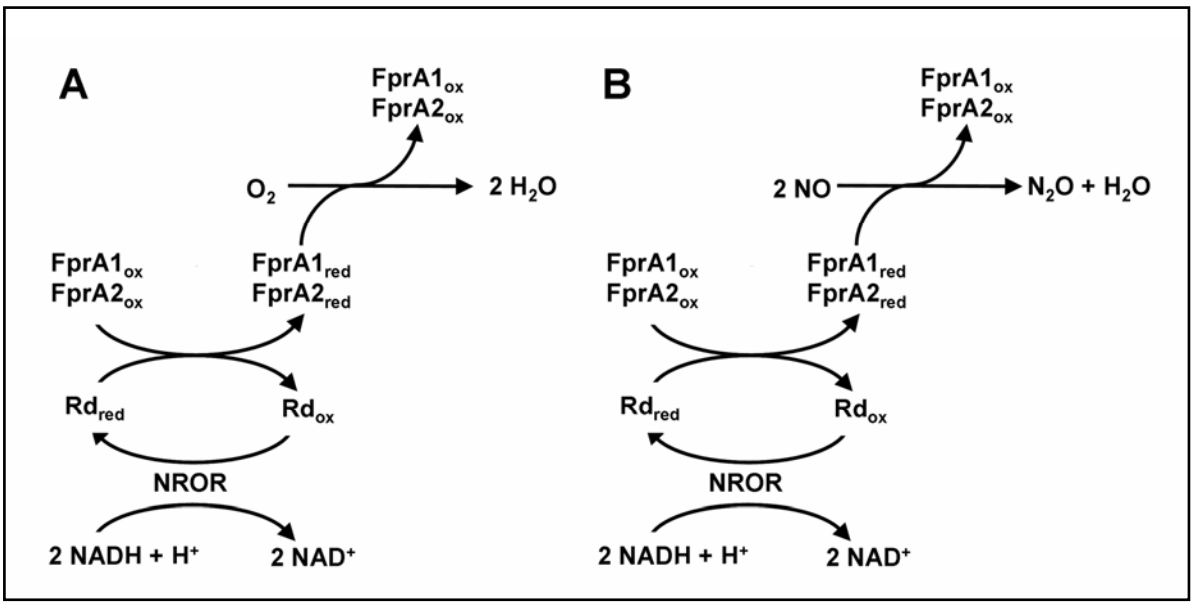

Abb. 10: Reduktive Detoxifizierung von O_2 (A) und NO (B) durch FprA1 und FprA2

FprA1 und **FprA2,** Flavo-2Fe-Proteine FprA1 und FprA2; **NROR**, NADH:Rubredoxin Oxidoreduktase; **Rd**, Rubredoxin; **ox**, oxidiert; **red**, reduziert.

Die spezifische Äktivität mit O_2 lag ca. dreifach höher als mit NO und war jedenfalls *in vitro* sogar 50-100fach höher als die des revRbr. Es ist sehr wahrscheinlich, dass die drei Proteine auch *in vivo* mit O_2 reagieren und so bei mirkoaerobem Wachstum Anaerobiose aufrechterhalten oder diese wiederherstellen, wenn kein weiterer O_2 zugeführt wird. Dennoch würden erst gezielte Mutationen der FDP kodierenden Gene (*fprA1, fprA2*) und des *rbr3A*/*B*-Operons die Frage beantworten, wie hoch der Beitrag der einzelnen Enzyme tatsächlich ist. Zur Mutation von *fprA1* oder *fprA2* sind bisher keine Versuche unternommen worden. Die innerhalb dieser Arbeit ebenfalls angestrebte, vollständige Deletion des *rbr3A/B*-Operons mittels homologer Rekombination blieb erfolglos. Auch durch das von HEAP *et al.* (2007) entwickelte „ClosTron"-System konnte bisher ein funktioneller „knock out" der beiden Zwillingsgene nicht zweifelsfrei nachgewiesen werden (RIEBE, 2009). Es ist nicht auszuschließen, dass es sich bei *rbr3A* und *rbr3B* zumindest unter Laborbedingungen um essentielle Gene handelt. Dafür spricht, dass die Transkription auch in Abwesenheit von äußeren Stressfaktoren vergleichsweise hoch ist (MAY *et al.*, 2004; HILLMANN, 2005). Hier böte sich methodisch z. B. ein „knock down" mittels antisense-RNA an (TUMMALA *et al.*, 2003). Alternativ wäre auch die Herstellung einer konditionellen revRbr-Mutante denkbar. Hierbei müsste mittels homologer Rekombination die Promoterregion von *rbr3A/B* gegen einen schwachen oder bestenfalls induzierbaren Promotor ausgetauscht werden.

Eine weitere offene Frage ist, welche Bedeutung der NO-Reduktase-Funktion beider Gene zukommt. Bekannte Ursachen für nitrosativen Stress sind die Argininoxidation durch die NO-Synthase in Makrophagen, sowie die Nitrat- und Nitritreduktion (STORZ und IMLAY, 1999; RODRIGUES *et al.*, 2006). Eine physiologische Verbindung zwischen NO und ROS wurde bereits aufgrund der NO-abhängigen Inaktivierungen der Transkriptionsfaktoren FNR und Fur postuliert (CRUZ-RAMOS *et al.*, 2002; D'AUTRÉAUX *et al.*, 2002). Reaktive Stickstoffverbindungen wie Peroxinitrit (HOONO) und Nitrosothiole (R-SNO) verweisen auch auf die funktionellen Ähnlichkeiten zwischen nitrosativem und oxidativem Stress (STORZ und IMLAY, 1999). Zum besseren Verständnis der Bedeutung der NO-Reduktaseaktivität *in vivo* wären Experimente zum nitrosativem Stress zwingend notwendig. Diese sollten zunächst klären, ob z. B. neben O_2 auch NO die Expression von *fprA1* und *fprA2* induziert. Wie wirken sich Defektmutationen von FprA1 und FprA2 auf die NO-Toleranz aus? In *D. gigas* führte die Abwesenheit eines funktionellen FDPs zu einer erhöhten Empfindlichkeit gegenüber NO, während O_2 nur einen geringen Effekt hatte (RODRIGUES *et al.*, 2006). Die O_2-abhängige Expression von FprA1 und FprA2 provoziert die Frage, ob in

C. acetobutylicum möglicherweise eine zwingende physiologische Verbindung zwischen O_2-Exposition und nitrosativem Stress besteht. Die Entstehung von NO unter mikroaeroben Wachstumsbedingungen könnte dabei z. B. durch den Farbstoff 4,5-Diaminofluorescin nachgewiesen werden (RÄTHEL *et al.*, 2003).

Innerhalb dieser Arbeit konnten mittels Transkriptomanalysen mikroaerob wachsender Zellen auch die O_2-induzierte Expression weiterer Peroxidasen gezeigt werden, die ebenfalls zur reduktiven Detoxifizierung in *C. acetobutylicum* beitragen können. Die putativen Proteinprodukte zeigen hohe Sequenzähnlichkeiten zu Thiolperoxidasen (CAC0327 und CAC3306*)* oder Glutathionperoxidasen (CAC1549, CAC1570 und CAC1571). In aeroben Organismen sind diese Proteine bereits ausführlich untersucht worden (CHAE *et al.*, 1994; MOORE und SPARLING, 1996; SIES *et al.*, 1997). Für *C. acetobutylicum* liegen aber bisher keine speziellen Kenntnisse zur Funktion dieser Proteine oder zu Thioredoxin/Glutaredoxin-abhängigen Systemen vor.

Die starke O_2-abhängige Expression sämtlicher reduktiver Detoxifikationsenzyme soll nicht darüber hinwegtäuschen, dass es keine Alternative zur Reduktion gäbe: Es existieren in *C. acetobutylicum*, sowie in vielen anderen Anaeroben, durchaus Gene, deren Proteinprodukte wahrscheinlich Dismutationen katalysieren können. Es war daher zunächst überraschend, dass unter O_2 die Transkription der beiden im Genom annotierten Superoxiddismutase-Gene, *sodC* (Cu/Zn-Typ, *cac1363*) und *sodB* (Fe/Mn-Typ, *cac2567*) kaum beeinflusst war. In keinem der 3 unabhängigen Microarray-Experimente konnte eine signifikant erhöhte Transkription der beiden Gene in Gegenwart von O_2 festgestellt werden. Keine, oder im Vergleich zu anderen Genen des O_2-Stimulons nur geringe, Induktionen von SODs wurden ebenfalls für *C. perfringens* und *D. vulgaris* festgestellt (GEISSMANN *et al.*, 1999; JEAN *et al.*, 2004; ZHANG *et al.*, 2006). Warum also werden von Anaeroben unter O_2 bevorzugt reduktive Enzyme verwendet? Obwohl die Gründe hierfür bisher noch nicht genau geklärt sind, ergeben sich für Anaerobe aus der NADH-abhängigen Reduktion von O_2 und ROS einige Vorteile (JENNEY *et al.*, 1999; IMLAY, 2002; IMLAY, 2008a; **Abb. 11**).

1. Die Reduktion von O_2^- und H_2O_2 setzt im Gegensatz zur Dismutation keinen zusätzlichen, toxischen O_2 frei.
2. Aktiven Gärern stehen Reduktionsäquivalente wie NADH in der exponentiellen Wachstumsphase in ausreichender Menge zur Verfügung.
3. Das alternative Detoxifikationssystem ermöglicht eine vereinfachte Regeneration von NAD^+ als Redoxpartner in der Glykolyse.

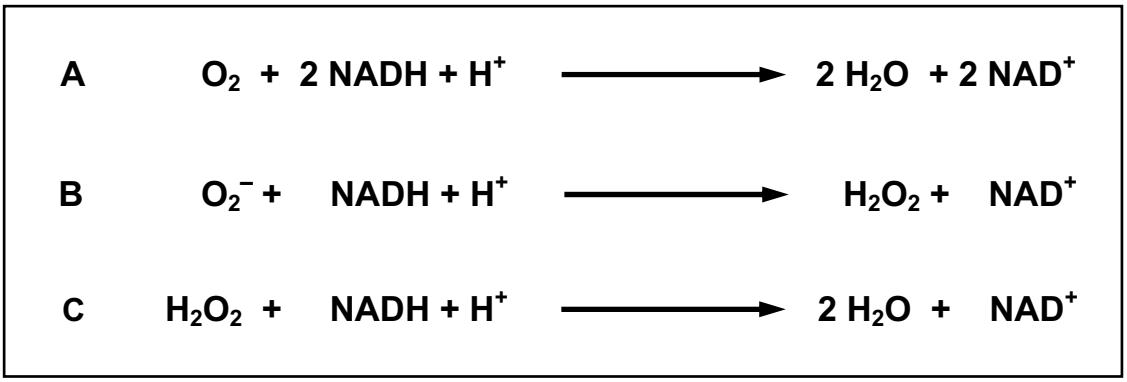

Abb. 11: NADH Oxidation während der Reduktion von O_2 (A), O_2^- (B) und H_2O_2 (C)

Statt die Elektronen wie üblich vom NADH unter Bildung der typischen Fermentationsprodukte wieder auf das Substrat zu übertragen, können diese alternativ zur Reduktion von O_2 oder ROS verwendet werden. Sicherlich spielt die Fähigkeit zur reduktiven Detoxifikation von O_2 und ROS eine entscheidende Rolle bei der Durchquerung und Besiedlung mikroaerober Lebensräume. Dennoch, gerade das letztgenannte Argument warf die Frage auf, ob neben der Beseitigung von O_2 und ROS auch Mechanismen zur Adaptation des Energiestoffwechsels existieren.

4.2.3 Energiestoffwechsel

Die massive Expression von O_2- und ROS-reduzierenden Enzymen hatte zwar einen entscheidenden Anteil am mikroaeroben Wachstum von *C. acetobutylicum,* befähigte jedoch nicht zu einer vollständigen Aerotoleranz. Bei starker Belüftung, die zur Anreicherung von O_2 im Flüssigmedium führte, kam es innerhalb von wenigen Minuten zu einem Stillstand des Wachstums und rapidem Abfall der LZZ. Eine Erklärung dieses Phänomens liefert möglicherweise der zentrale Energiestoffwechsel von *C. acetobutylicum*. Die Pyruvat-Ferredoxin Oxidoreduktase (PFOR) katalysiert die Oxidation des Pyruvats zu Acetyl-CoA bei gleichzeitiger Reduktion von Ferredoxin. Der PFOR wurde eine hohe Sensibilität gegenüber O_2 attestiert (MEINECKE *et al.*, 1989). Obwohl das beschädigte Enzym bisher nicht genau untersucht wurde, wird vermutet, dass O_2 ein äußeres Fe-S-Cluster zerstört, was zur oxidativen Inaktivierung des Enzyms führt (CHARBRIERE *et al.*, 1999; IMLAY, 2006). Tatsächlich konnte bereits *in vivo* gezeigt werden, dass O_2 zur Inaktivierung der PFOR von *B. thetaiotoamicron* führt (PAN und IMLAY, 2001).

Einige Beobachtungen sprechen dafür, dass die PFOR in *C. acetobutylicum* ein limitierender Faktor der O_2-Toleranz ist: Eine verringerte Aktivität der PFOR würde sich womöglich schnell auf den intrazellulären Pool von Acetyl-CoA auswirken. Acetyl-CoA ist u. a. ein essentieller Baustein in der Synthese von Phospholipiden und damit ein entscheidender Faktor für die Zellteilung. Interessanterweise war im lichtmikroskopischen Bild ein Großteil der Zellen (25-50 %) auch unter O_2 längere Zeit motil, selbst dann, wenn die LZZ bereits unter 0,1% lag (unveröffentlichte Daten). Die anhaltende Motilität verweist möglicherweise auf eine funktionierende Glykolyse, die noch kontinuierlich ATP liefert, während ein Mangel an Acetyl-CoA jedoch Zellteilungen verhindert. Die O_2-labile PFOR könnte also frühere Beobachtungen erklären, nach denen *C. acetobutylicum* in Gegenwart von O_2 zunächst das Wachstum einstellt aber „weiterlebt“ (O'BRIEN und MORRIS, 1971).

Die innerhalb dieser und anderer Arbeiten verwendeten aeroben Schüttelkulturen führten allerdings sofort zu einem rapiden Absinken der LZZ. Hier ist es durchaus denkbar, dass der schockartige Transfer anaerober, metabolisch aktiver Zellen in O_2-gesättigtes Medium schneller zu letalen H_2O_2- und O_2^--Produktionsraten führte, als eine effiziente Adaptation stattfinden konnte. Dass dabei die durch H_2O_2 angetriebene Fentonreaktion tatsächlich zum Absterben der Zellen führte, konnte durch die Zugabe des Eisenchelators Deferrioxamin gezeigt werden. Deferrioxamin bindet intrazelluläres, freies Fe^{2+} und verhindert so die Bildung von Hyxdroxylradikalen (HO•), der reaktivsten O-Verbindung (IMLAY *et al.*, 1988; **Abb. 6**). Tatsächlich bewirkte auch bei *C. acetobutylicum* die Zugabe von Deferrioxamin eine gesteigerte LZZ unter O_2. Um so deutlicher war aber der Unterschied zur *perR* Deletionsmutante: Die Zellen starben nicht (>6 h), sondern behielten sogar ein zeitlich limitiertes Wachstum bei (2 h). Letztgenannter Effekt ist durch das reduktive Detoxifizierungssystem nicht erklärbar, denn es wurde so stark belüftet, dass die Konzentration des gelösten O_2 fast den Sättigungswert (~260 µM) erreichte. Dabei ist die Diffusionsrate von O_2 über Zellmembranen so hoch, dass die intra- und extrazellulären O_2-Konzentrationen nahezu identisch sind (LIGEZA *et al.*, 1998). Es lag also die Vermutung nahe, dass über eine effiziente Reduktion von O_2 und ROS hinaus weitere Mechanismen existieren die ein Überleben in Gegenwart von O_2 ermöglichen. Obwohl es hierzu bisher insgesamt nur wenige Erkenntnisse gibt, konnten die durchgeführten Transkriptomanalysen zeigen, dass sehr wahrscheinlich weitere Überlebensstrategien bei O_2-Stress zur Verfügung stehen. Es sei hier auch auf die Diskussionen der einzelnen Publikationen hingewiesen. Im Folgenden soll daher nur auf die putativen Modifizierungen des zentralen Stoffwechselwegs eingegangen werden (**Abb. 12**).

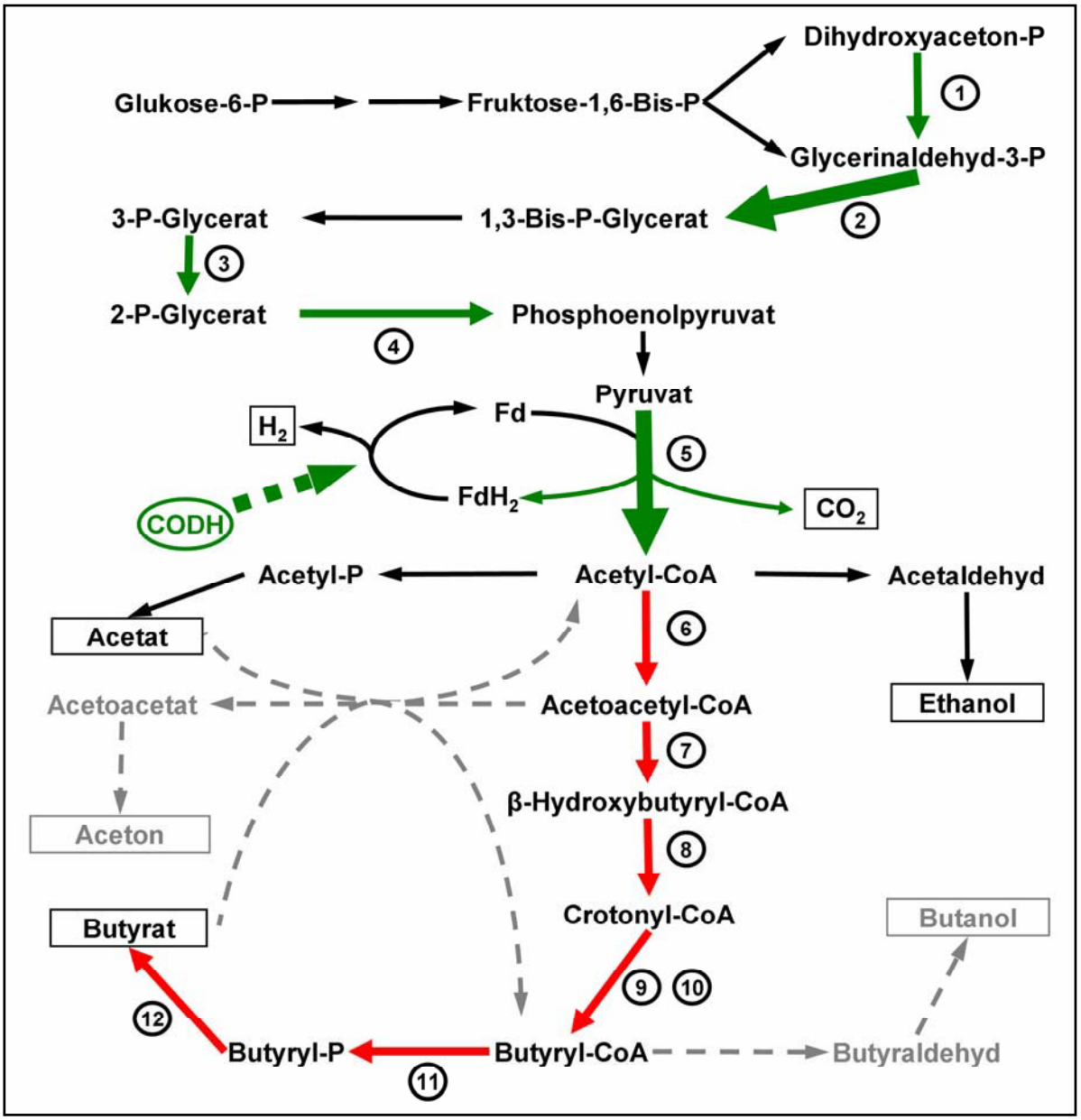

Abb. 12: Regulation des Gärungsstoffwechsels von *C. acetobutylicum* unter O_2.

Innerhalb dieser Arbeit wurden nur Zellen aus der Säure-bildenden Wachstumsphase analysiert. Hauptreaktionen der Lösungsmittelbildung sind durch graue, gestrichelte Pfeile gekennzeichnet. Stoffwechselendprodukte sind eingerahmt. Enzyme mit unveränderter (schwarz), erhöhter (grün) und reduzierter Expression (rot) unter O_2 sind als Pfeile dargestellt. Die Funktion der stark induzierten Kohlenmonoxid-Dehydrogenase ist spekulativ (CODH, Induktionsfaktor 23,9). Die Größe der Pfeile kennzeichnen die relative Induktion bzw. Reduktion der Transkription unter O_2. **1**: Triosephosphatisomerase, Induktionsfaktor 2,1; **2**: NADP-abhängige-GAPDH, 13,6; **3**: Phosphoglyceromutase, 2,8; **4**: Enolase, 3,3; **5**: „oxo-acid"-Ferredoxin-Oxidoreduktase, 9,2; **6**: Thiolase, 0,31; **7**: Hydroxybutyryl-CoA-DH, 0,34; **8**: Crotonase, 0,28; **9**: Butyryl-CoA-DH, 0,28; **10**: Elektronentransfer-Flavoproteine A und B, 0,29 bzw. 0,26; **11**: Phophotransbutyrylase, 0,38; **12**: Butyratkinase, 0,41

4.2.3.1 Eine „oxo-acid"-Ferredoxin-Oxidoreduktase (OFOR)

Für zwei Gene *(cac2458/9)*, die wahrscheinlich die α- und β-Untereinheiten eines PFOR-ähnlichen Enzyms kodieren, konnte sowohl unter O_2 (beide Faktor 9) als auch in der *perR* Mutante (Faktor 24 bzw. 40) eine massive Induktion der Transkription festgestellt werden. Die Stärke des Fluoreszenzsignals für beide Gene im Microarray erreichte etwa 60 % des Wertes für die mRNA des Elongationsfaktors Tu (EfTu). Dies weist auf ein äußerst stark exprimiertes Operon hin. Das Produkt ist bisher nicht näher charakterisiert worden, es wäre jedoch vorstellbar, dass dieses Enzym, wenn auch mit geringerer Spezifität, an der Ferredoxin-abhängigen Pyruvatoxidation beteiligt ist. Interessanterweise werden ähnliche Enzyme auch von einigen aeroben Archaeen verwendet, wie bereits für *Sulfolobus sp.* und *Aeropyrum pernix* gezeigt werden konnte (ZHANG *et al.*, 1996; NISHIZAWA *et al.*, 2005). Auch die O_2-Labilität von PFORs ist keinesfalls von universeller Gültigkeit. So besitzt z. B. *D. africanus* eine O_2-stabile PFOR, bei der das terminale Fe-S-Cluster durch eine zusätzliche Domäne vor oxidativer Inaktivierung geschützt wird (PIEULLE *et al.*, 1997). In *B. thetaiotaomicron* existieren sogar spezielle Reparaturmechanismen, die nach Wiederherstellung der Anaerobiose oxidativ beschädigte Enzyme, wie die PFOR, reaktivieren (PAN und IMLAY, 2001). Eine biochemische Charakterisierung der (rekombinanten) OFOR aus *C. acetobutylicum* könnte hier Aufschluss über funktionelle Eigenschaften, wie z. B. das Substratspektrum und die O_2-Stabilität, geben.

4.2.3.2 Eine NADP-abhängige Glycerinaldehyd-3-Phosphat-Dehydrogenase

Ähnlich stark wie die beiden Gene der putativen OFOR wird das einer NADP-abhängigen Glycerinaldehyd-3-Phosphat-Dehydrogenase (NADP-GAPDH, *cac3657*) unter O_2 transkribiert (Induktionsfaktor 13; Signalstärke ca. 90 % der EfTu mRNA). Auch dieses Gen wird direkt durch PerR reguliert. Die Aktivität und NADP-Spezifität dieses Enzyms wurden erst kürzlich experimentell bestätigt (MARTINEZ *et al.*, 2008). Auch die Gene für die Enzyme, die die Umwandlung von Dihydroxyacetonphophat zu GAP (Triosephosphatisomerase, *cac0711*) bzw. die zwei der GAPDH nachfolgenden Reaktionen katalysieren, (Phosphoglyceromutase, *cac0712* und Enolase, *cac0713*) werden unter O_2 stärker transkribiert. Hier liegt möglicherweise eher ein Sekundäreffekt durch die starke Expression der NADP-GAPDH vor, denn in der anaeroben *perR*-Mutante ist die Transkription des Operons noch stärker, obwohl es nicht direkt durch

PerR reguliert wird. Wahrscheinlich ermöglicht die verstärkte Expression der NADP-GAPDH unter O_2 ein Aufladen des NADPH-Pools, der von essentieller Bedeutung für die kontinuierliche Reduktion von Thioredoxin und Gluthathion ist (HENSLEY *et al.*, 2000; NORDBERG und ARNER, 2001).

4.2.3.3 Die Enzyme des Butyratwegs

Bei *C. acetobutylicum* ist während der exponentiellen Wachstumsphase in Batch-Kulturen Butyrat neben Acetat das Hauptgärungsprodukt. Durch Butyratbildung werden, ausgehend von 1 mol Acetyl-CoA, 2 mol NAD^+ Reduktionsäquivalente regeneriert. In Gegenwart von O_2 waren die Gene aller 8 Proteine, die an der schrittweisen Umwandlung von Acetyl-CoA zu Butyrat beteiligt sind, um den Faktor 2,4-3,6 schwächer exprimiert (**Abb. 12**). Auch wenn der Faktor der Reduktion im Vergleich zu denen der induzierten Gene eher gering war, kann dennoch davon ausgegangen werden, dass die moderate Repression dieser Gene ein indirekter Effekt des O_2 ohne PerR-Regulation ist. Folgende Argumente unterstützen dieses Ergebnis:

1. Eine schwächere Expression aller Gene war in allen 3 unabhängigen Experimenten nachweisbar.
2. In der PerR-Deletionsmutante wurde unter anaeroben Bedingungen keines der Gene schwächer transkribiert.
3. Die Gene befinden sich auf dem DNA-Minusstrang an 3 weit voneinander entfernten genomischen Loci (**Abb. 13**).

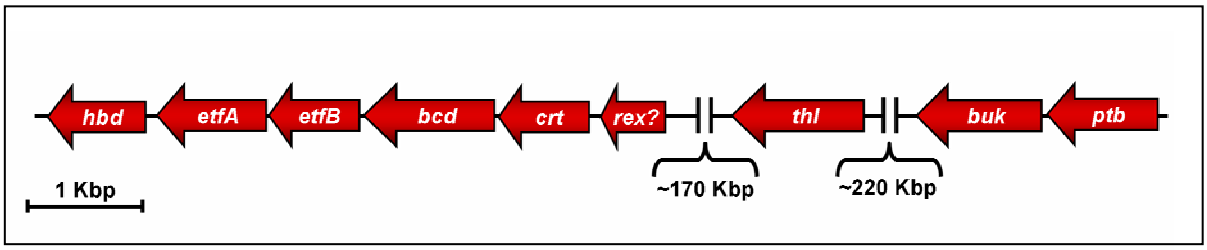

Abb. 13: Genomische Organisation der Gene des Butyratstoffwechsels

hbd: Hydroxybutyryl-CoA-Dehydrogenase, *cac2708*; ***etfA*** und ***etfB***: Elektronentransfer-Flavoproteine A und B, *cac2709* und *cac2710*; ***bcd***: Butyryl-CoA-Dehydrogenase, *cac2711*; ***crt***: Crotonase, *cac2712*; ***rex*****:** putativer Redoxregulator, *cac2713*; ***thl***: Thiolase, *cac2873*; ***buk***: Butyratkinase, *cac3075*; ***ptb***: Phosphotransbutyrylase, *cac3076*

Die Gründe für die moderate Diskriminierung des Butyratweges sind bisher noch nicht näher untersucht worden. Bedenkt man aber, dass auf diesem Weg die meisten Reduktionsäquivalente regeneriert werden, so ist es durchaus vorstellbar, dass bei mikroaerobem Wachstum dieses auch mit O_2 oder H_2O_2 als alternativen Elektronenakzeptoren erreicht werden könnte. So würde die Reduktion von O_2 das Redoxgleichgewicht aus NAD^+ und NADH zugunsten des NAD^+ verschieben. Verbindungen zwischen NADH/NAD^+-Ratio und Veränderungen des Produktspektrums von *C. acetobutylicum* wurden bereits früher beobachtet und legten die Schlussfolgerung nahe, dass erstere an der Regulation der Butyrat- und Butanolproduktion beteiligt ist (MEYER und PAPOUTSAKIS, 1989; VASCONCELOS *et al.*, 1994; GIRBAL und SOUCAILLE, 1994).

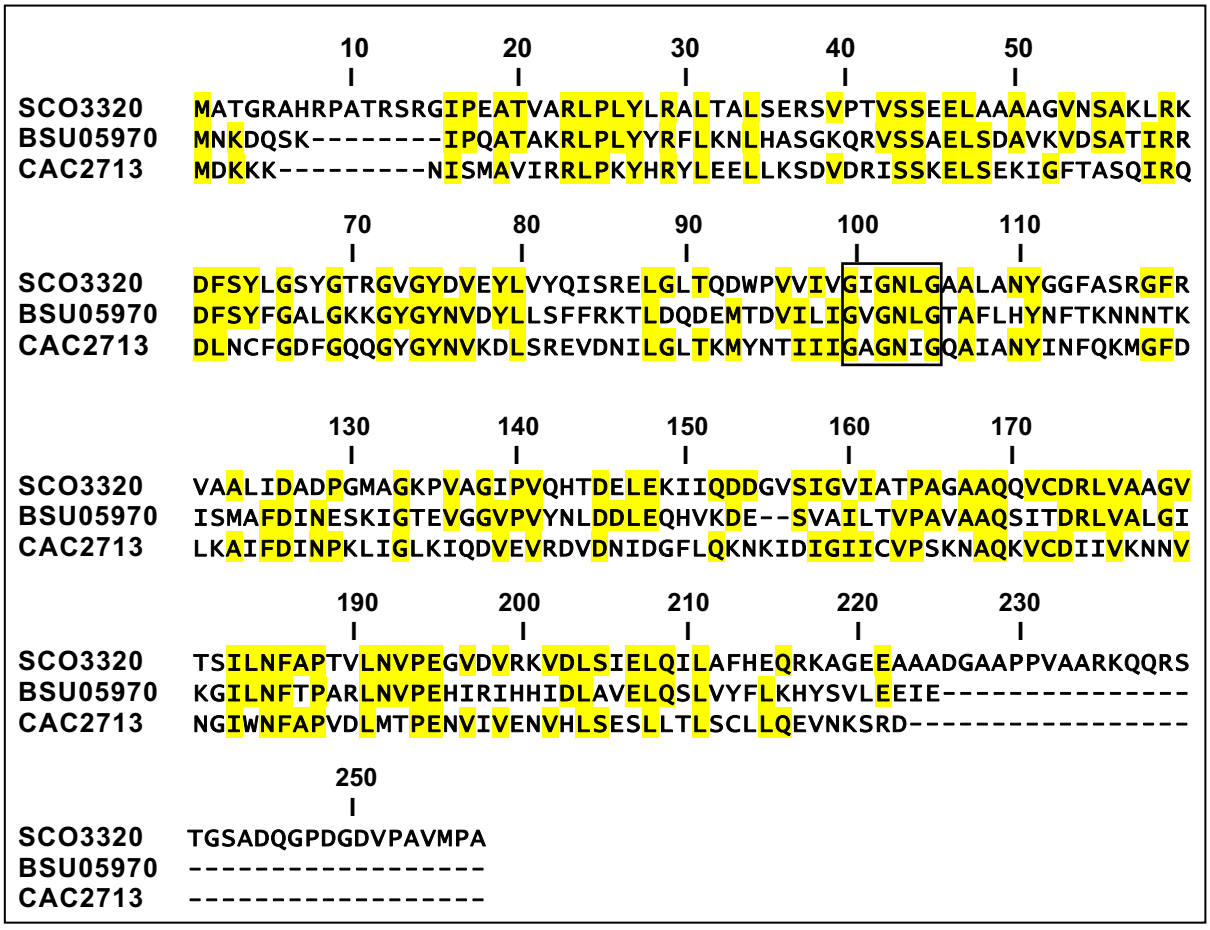

Abb. 14: Sequenzvergleich NADH/NAD^+ Redoxregulator (Rex)-ähnlicher Proteine

Zahlen oberhalb der Sequenz kennzeichnen die Position der Aminosäuren. Übereinstimmende Sequenzbereiche sind gelb hinterlegt. Das konservierte **G-X-G-XX-G**-Motiv ist eingerahmt. **SCO3320**: Rex aus *S. coelicolor* (BREKASIS und PAGET, 2003); **BSU05970**: Rex aus *B. subtilis* (WANG et al., 2008): **CAC2713**: putatives Rex-Protein aus *C. acetobutylicum*.

Es wäre daher sicherlich interessant zu erfahren ob bei mikroaeroben Wachstum vornehmlich Produkte entstehen, die weniger reduziert sind als Butyrat oder Butanol. Ein möglicher experimenteller Ansatz hierzu wäre die mikroaerobe Kultivierung von *C. acetobutylicum* im Chemostaten mit definierter O_2-Zufuhr und Analyse der Gärungsprodukte. Wahrscheinlich ist bei der Regulation des Butyratweges auch der ORF *cac2713* von entscheidender Bedeutung. Dieser ORF ist in unmittelbarer Nachbarschaft zu einigen Genen des Butyratweges lokalisiert (**Abb. 13**). Das putative, von *cac2713* kodierte Protein zeigt sowohl strukturelle Ähnlichkeiten zu bereits charakterisierten NADH/NAD^+-Redoxregulatoren aus *S. coelicolor* und *B. subtilis*, als auch das konservierte Aminosäuremotiv GXGXXG, das von essentieller Bedeutung für ein „redox-sensing“ ist (BREKASIS und PAGET, 2003; WANG et al., 2008, **Abb. 14**).

4.2.3.4 Ein Kohlenmonoxid-Dehydrogenase ähnliches Protein (*cac0116*)

In Gegenwart von O_2 war die Transkription eines Gens dessen Proteinprodukt hohe Sequenzähnlickeiten zu Kohlenmonoxid Dehydrogenasen (CODH) zeigt (**Tab. 4**), äußerst stark induziert (Faktor ca. 24). Es handelte sich dabei um ein vergleichsweise stark exprimiertes Gen, da die Fluoreszenzsignale bei über 100 % des Signals für die mRNA von EfTu liegen und das Proteinprodukt auch in 2-dimensionalen Proteingelen nachgewiesen werden konnte (FIEDLER, 2006). Auf die gegensätzliche Regulation in Abwesenheit von PerR wurde bereits unter 4.1.3 eingegangen. Da bisher keine Kenntnisse zu CODHs in *C. acetobutylicum* vorliegen, kann über eine mögliche Funktion dieses Proteins nur spekuliert werden. CODHs wurden bisher verschiedene Funktionen zugeschrieben, dabei sind besonders die Energiekonservierung durch den CO-vermittelten Aufbau von Protonengradienten (SOBOH *et al.*, 2002) und die Fixierung von CO_2 im reduktiven Acetyl-CoA Weg (ROBERTS *et al.*, 1989) hervorzuheben. Zu diesen Enzymen bestehen seitens des homologen Proteins aus *C. acetobutylicum* allerdings nur sehr geringe Sequenzähnlichkeiten (**Tab. 4**). Es ist daher zum jetzigen Zeitpunkt auch nicht auszuschließen, dass die CODH aus *C. acetobutylicum* in Gegenwart von O_2 direkt an der Reduktion von Rubrerythrinen beteiligt ist, was aufgrund der genomischen Lokalisation auch schon für die CODH-IV aus *Carboxydothermus hydrogenoformans* postuliert wurde (WU *et al.*, 2005). Es ist aber auch denkbar, dass die Aktivität der CODH das äußerst O_2-labile aktive Zentrum der Hydrogenasen von *C. acetobutylicum* (DEMUEZ *et al.*, 2007) durch den kompetetiven Inhibitor CO vor einer oxidativen und irreversiblen Inaktivierung schützt

(MANESS *et al.*, 2002). Für die sehr sequenzhomologe CODH (76 % identische Aminosäuren, **Tab. 4**) aus *C. kluyveri* ist kürzlich vermutet worden, dass diese durch die Bereitstellung oder Beseitigung von CO-Liganden an der Synthese oder dem Abbau von [Fe-Fe]-Hydrogenasen beteiligt ist (SEEDORF *et al.*, 2008). Zur *in vivo* Funktion dieses Proteins unter O_2 wären weitere Arbeiten unbedingt empfehlenswert.

Tab. 4: Vergleich der Aminosäuresequenz des ORFs CAC0116 mit bekannten Proteinen[1]

ORF	Mögliche Funktion[2]	Aminosäureidentität /-ähnlichkeit zu *CAC0116* (%)
M. thermoacetica		
MOTH1198	Acetyl-CoA-Synthase δ-Untereinheit	11 / 17
MOTH1201	Acetyl-CoA-Synthase γ-Untereinheit	16 / 26
MOTH1202	Acetyl-CoA-Synthase α/β-Untereinheit	16 / 27
MOTH1203	CODH?	27 / 47
MOTH1972	CODH?	29 / 52
Ca. hydrogenoformans		
CHY_0249	Acetyl-CoA-Synthase (CODH-III)	6 / 10
CHY_1222	Acetyl-CoA-Synthase (CODH-III)	12 / 22
CHY_0085	Reduktion von NADP? (CODH-II)	30 / 50
CHY_0736	oxidative Stressantwort? (CODH-IV)	31 / 50
CHY_1824	Energiekonservierung? (CODH-I)	33 / 50
CHY_0034	? (CODH-V)	33 / 52
Cl. kluyveri		
CKL_2148	Hydrogenase Synthese / Abbau ?	76 / 89

[1] Die Aminosäuresequenzen von CAC0116 aus *C. acetobutylicum* und ORFs aus *Carboxydothermus hydrogenoformans* (WU *et al.*, 2005), *Moorella thermoacetica* (PIERCE *et al.*, 2008) und *Clostridium kluyveri* (SEEDORF *et al.*, 2008) wurden mit der „EMBOSS align" Software verglichen (RICE *et al.*, 2000).

[2] nach WU *et al.* (2005), PIERCE *et al.* (2008) und SEEDORF *et al.* (2008)

4.3 Ein verbessertes Modell zur obligaten Anaerobiose

Die Bezeichnungen „obligat“ und „strikt“ anaerob werden häufig synonym für eine sehr heterogene Gruppe von Prokaryoten verwendet, die in Gegenwart bestimmter O_2-Konzentrationen kein Wachstum zeigt. Insbesondere das Attribut „strikt“ geht dabei wahrscheinlich auf historische Beobachtungen zurück und wurde generell für Organismen verwendet, die bei atmosphärischen O_2-Konzentrationen gar nicht wachsen (QUASTEL und STEPHENSON, 1926; STICKLAND, 1934; HIRANO *et al.*, 1957). Die molekularen Grundlagen dieses Phänomens waren bis vor kurzem wenig bekannt. Eine vergleichende Studie über die Abwesenheit von Katalasen und Superoxiddismutasen (SOD) in solchen Organismen bildete lange Zeit die Grundlage des gängigen Modells, dass eben die Abwesenheit dieser Enzyme das Leben in anaerobe Habitate zwingt (McCORD *et al.*, 1971). Clostridien galten als klassische Vertreter dieser Gruppe, denn sowohl Superoxiddismutase- als auch Katalaseaktivität waren kaum oder gar nicht messbar (GREGORY *et al.*, 1978; BRIOUKHANOV *et al.*, 2002).

Die Aerotoleranz der *perR*-Deletionsmutante widerlegte das postulierte Modell McCORDs *et al.* (1971), das bereits zuvor u. a. durch die Entdeckung der Superoxidreduktase und O_2-empfindlicher Enzyme des Gärungstoffwechsels hinterfragt worden war (JENNEY *et al.*, 1999; PAN und IMLAY, 2001). Darüber hinaus lässt sich erkennen, dass *C. acetobutylicum* mehrere physiologische Möglichkeiten zur Verfügung stehen, um sich an mikroaerobe Wachstumsbedingungen zu adaptieren. Es scheint jedoch, als erfüllten dabei insbesondere das reduktive Detoxifikationssystem und das übrige PerR Regulon zentrale Funktionen, die über die bloße Entfernung des O_2 oder ROS hinausgehen könnten. Über das Ausmaß der physiologischen Veränderungen bei einer Verwendung von O_2 als alternativem Elektronenakzeptor, kann derzeit nur spekuliert werden. Tiefergehende Kenntnisse der *in vitro* und *in vivo* Funktionen der zuvor genannten Proteine unter O_2, würden sicherlich nicht nur zu einem erweiterten Verständnis der O_2-Stressantwort, sondern auch der Regulation zentraler Stoffwechselwege führen.

Die Klassifizierung als „obligat anaerob“ bleibt insofern berechtigt, als dass unter Laborbedingungen atmosphärische O_2-Konzentrationen ein Wachstum des Wildtyps verhindern. Letztendlich sind es aber wahrscheinlich nur einige wenige Stoffwechselreaktionen die jedenfalls bis heute eine vollständige Aerotoleranz

verhindert haben. Es ist zumindest vorstellbar, dass einige „obligate Anaerobier“ unter physiologischen Bedingungen, wie z. B. einem sehr allmählichen Anstieg der O_2-Konzentration, den Grad der Anpassung und die Aerotoleranz der *perR*-Mutante von *C. acetobutylicum* noch weit übertreffen könnten.

5 ZUSAMMENFASSUNG

1. Das in *C. acetobutylicum* ursprünglich nach einem Hitzeschock identifizierte Hsp21 ist ein Rubrerythrin mit reverser Domänenstruktur, für das erst kürzlich durch RIEBE *et al.* (2009) eine Rubredoxin-abhängige Peroxidase- und O_2-Reduktase-Funktion beschrieben wurde. Es wird durch die beiden Zwillingsgene *rbr3A* und *rbr3B* kodiert und als bicistronische Transkriptionseinheit exprimiert. Die Transkription der Gene konnte neben einem Hitzeschock auch durch weitere, insbesondere aber durch oxidative Stressfaktoren induziert werden.

2. Nach einer Analyse der Promotorregion des *rbr3*-Operons konnte in der 5'UTR ein AT-reiches Sequenzpalindrom identifziert werden. Aufgrund der Konservierung dieses Palindroms vor oxidativen Stressgenen im Genom von *C. acetobutylicum* und anderen Clostridien wurde eine Rolle als *cis*-regulatorisches Element postuliert.

3. Ein PerR- (Peroxid-„Response" Regulator) ähnliches Protein wurde als Trankriptionsrepressor des *rbr3*-Operons identifiziert. Dies konnte zunächst indirekt durch Überexpression des Hsp21 in *perR* mutierten Zellen und anschließend durch die spezifische Bindung heterlogen PerRs and die *rbr3*-Promotorregion gezeigt werden.

4. *B. subtilis perR*-Defektmutanten konnten durch das *perR*-Gen aus *C. acetobutylicum* komplementiert werden. Durch induzierte Expression des homologen Proteins aus *C. acetobutylicum* wurde der peroxidresistente Phänotyp der *B. subtilis perR*-Defektmutante revertiert. Es konnten hierdurch die Schlußfolgerungen gezogen werden, dass es sich tatsächlich um ein funktionell konserviertes PerR-Protein handelt und dass *C. acetobutylicum* H_2O_2 als sensorischen Marker für O_2 verwendet.

5. Die Deletion des *perR*-Gens in *C. acetobutylicum* bewirkte eine deregulierte Expression aller bisher bekannten Komponenten des alternativen ROS-Detoxifikationsystems von anaeroben Mikroorganismen, was durch die massiv erhöhten Reduktionsaktivitäten für O_2^- und H_2O_2 bestätigt werden konnte. Die Mutante besaß eine ebenso stark erhöhte O_2-Verbrauchsrate.

6. Es wurden hierzu die zwei funktionell unbekannten Flavoproteine FprA1 und FprA2 biochemisch charakterisiert. Beide gehören zur Familie der Flavo-2Fe-Proteine und katalysierten *in vitro* die Rubredoxin-abhängige Reduktion von O_2 mit ähnlichen Aktivitäten. Zusätzlich konnte für FprA2 eine Rubredoxin-abhängige NO-Reduktaseaktivität gemessen werden. Die Gene der beiden Proteine konnten ebenfalls dem PerR Regulon zugeordnet werden.

7. Die *perR*-Deletionmutante war aufgrund der vollständigen Aktivierung des PerR Regulons auch durch einen aerotoleranten Phänotyp charakterisert. Diese Ergebnisse konnten das von McCORD *et al.*, (1971) formulierte Modell, wonach obligate Anaerobiose eine Konsequenz geringer Katalase- und Superoxid-Dismutase Aktivitäten sei, endgültig widerlegen.

8. Durch globale Transkriptionsanalysen der Deletionsmutante und die genomweite Identifizierung putativer PerR-Bindestellen konnte das PerR-Regulon von *C. acetobutylicum* genauer definiert werden. Demzufolge sind neben dem alternativen Detoxifikationsystem auch alternative Enzyme zentraler Stoffwechselwege einer direkten Repression durch PerR unterworfen (NADPH-abhängige GAPDH und die α- und β-Untereinheit der einer OFOR). PerR ist aber nicht nur als Repressor aktiv, sondern besitzt auch eine noch nicht näher geklärte Funktion als Transkriptionsaktivator.

9. Globale Transkriptionsanalysen mit Zellen des Wildtyps unter mikroaeroben Wachstumsbedingungen zeigten, dass *C. acetobutylicum* neben dem PerR Regulon noch über weitere Adaptationsmechanismen für O_2 verfügt. Die Anwesenheit von O_2 führte u. a. zur Aktivierung eines Glutathion- und Thioredoxin abhängigen Systems zur Detoxifikation und Regulation des Redoxgleichgewichts. Zusätzlich ist *C. acetobutylicum* in der Lage den zentralen Energiestoffwechsel oxidativen Wachstumsbedingungen anzupassen. Dies war besonders anhand der reduzierten Expression von Genen des Butyratweges sichtbar.

6 LITERATURVERZEICHNIS

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7 ANHANG

7.1 Erklärungen

7.1.1 Anteilserklärung für Falk Hillmann

Hillmann, F., R.-J. Fischer und H. Bahl. 2006. The rubrerythrin-like protein Hsp21 of *Clostridium acetobutylicum* is a general stress protein. *Arch. Microbiol.* **185**:270-276.

- Durchführung aller experimentellen Arbeiten, Fluoreszenzmikroskopie unter Anleitung und mit Hilfe von Dr. Rhena Schumann
- Erstfassung des Manuskripts

Hillmann, F., R.-J. Fischer, F. Saint-Prix, L. Girbal und H. Bahl. 2008. PerR acts as a switch for oxygen tolerance in the strict anaerobe *Clostridium acetobutylicum. Mol. Microbiol.* **68**:848-860.

- Durchführung aller experimentellen Arbeiten, Konstruktion der *perR* Deletionsmutante von *C. acetobutylicum* am INSA in Toulouse, Frankreich mit Hilfe von Dr. F. Saint-Prix und unter Anleitung von Dr. L. Girbal
- Auswertung der experimentellen Daten
- Erstfassung des Manuskripts

Hillmann F., O. Riebe, R.-J. Fischer, J. Caranto, A. Mot, D. M. Kurtz Jr. und H. Bahl. 2009. Reductive dioxygen scavenging by flavodiiron proteins of *Clostridium acetobutylicum*. *FEBS Lett*. **583**:214-245.

- Sequenzvergleiche
- Konstruktion der FprA1 und FprA2 Überexpressionstämme
- Überexpression und Aufreinigung von rekombinanten FprA1 und FprA2
- NADH-Oxidase und O_2-Reduktase Messungen und Auswertung der Messergebnisse
- Erstfassung des Manuskripts

Hillmann F., C. Döring, A. Ehrenreich, R.-J. Fischer und H. Bahl. 2009. The role of PerR in O_2 affected gene expression of *Clostridium acetobutylicum. Manuskript in Vorbereitung*

- Durchführung aller experimentellen Arbeiten, mit Ausnahme der RNA Markierungen und anschließenden Chip-Hybridisierungen (C. Döring im Labor von A. Ehrenreich an der Universität Göttingen)
- Auswertung der Daten der Microarray-Experimente unter Anleitung und mit Hilfe von C. Döring
- Auswertung aller anderen Daten
- Erstfassung des Manuskripts

7.1.2 Selbstständigkeitserklärung

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Falk Hillmann Rostock, den 26.01.2009

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7.3 Publikationsliste

- **Hillmann, F.**, C. Döring, A. Ehrenreich, R.-J. Fischer und H. Bahl. 2009. The role of PerR in O_2 affected gene expression of *Clostridium acetobutylicum. Manuskript in Vorbereitung*

- **Hillmann, F.**, O. Riebe, R. J. Fischer, A. Mot, J. D. Caranto, D. M. Kurtz Jr. und H. Bahl. 2009. Reductive dioxygen scavenging by flavodiiron proteins of *Clostridium acetobutylicum. FEBS Lett*. **583**: 241-245.

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  Commented in: Imlay J. A. 2008. How obligatory is anaerobiosis? *Mol. Microbiol.* **68:** 801-804.

- **Hillmann, F.**, R. J. Fischer und H. Bahl. 2006. The rubrerythrin-like protein Hsp21 of *Clostridium acetobutylicum* is a general stress protein. *Arch. Microbiol.* **185:** 270-276.

- May A., **F. Hillmann**, O. Riebe, R. J. Fischer und H. Bahl. 2004. A rubrerythrin-like oxidative stress protein of *Clostridium acetobutylicum* is encoded by a duplicated gene and identical to the heat shock protein Hsp21. *FEMS Microbiol. Lett*. **238:** 249-254.

7.4 Tagungsbeiträge

- Mann, M. S., O. Riebe, **F. Hillmann** und H. Bahl. Microoxic growth and metabolism of *Clostridium acetobutylicum*. *Jahrestagung der VAAM 2009, Bochum* (Vortrag).

- Scheel, M., **F. Hillmann,** R.-J. Fischer und H. Bahl. The oxidative stress response of *Clostridium acetobutylicum*. *Jahrestagung der VAAM 2009, Bochum* (Poster).

- **Hillmann, F.**, R.-J. Fischer, F. Saint-Prix, L. Girbal und H. Bahl. PerR acts as a switch for oxygen tolerance in the strict anaerobe *Clostridium acetobutylicum*. *Jahrestagung der VAAM 2008, Frankfurt a. M.* (Vortrag).

- **Hillmann F.**, L. Girbal, C. Croux, R.-J. Fischer und H. Bahl. Construction of Hsp21 and Fur3 mutants of *Clostridium acetobutylicum* by chromosomal integration. *Jahrestagung der VAAM 2007, Osnabrück* (Poster).

- **Hillmann F.**, R.-J. Fischer und H. Bahl.. The rubrerythrin-like protein Hsp21 is a general stress protein. *Jahrestagung der VAAM 2006, Jena* (Poster).

- **Hillmann F.**, R.-J. Fischer und H. Bahl.. The rubrerythrin-like protein Hsp21 is a general stress protein. *Clostridium IX conference 2006 Houston, Texas, USA* (Poster).

- Riebe O., **F. Hillmann**, R.-J. Fischer und H. Bahl. The oxidative stress response of *Clostridium acetobutylicum. Jahrestagung der VAAM 2005, Göttingen* (Poster).

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7.5 Lebenslauf

☐ **Geburtsdatum:**

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Schulbildung

☐ **1989-1998**

Immanuel-Kant-Gymnasium in Neumünster (1989-1994)

Fritz-Reuter-Gymnasium in Kühlungsborn (1994-1998)

Washburn High School, Topeka, USA (1995-1996)

☐ **Juli, 1998**

Abitur in Kühlungsborn

☐ **1998-1999**

Wehrdienst in Heide (Schleswig-Holstein) und Jever (Niedersachsen)

Akademische Ausbildung

☐ **Oktober, 1999 - Febuar, 2005**

Biologiestudium an der Universität Rostock

☐ **August - September, 2001**

Vordiplom in den Fächern Physik, Chemie, Mikrobiologie, Genetik, Zoologie und Botanik

☐ **November, 2003 - Januar, 2004**

Diplomprüfungen in den Fächern Mikrobiologie (Hauptfach), Medizinische Mikrobiologie, Genetik und Molekularbiologie und Pflanzenphysiologie (Nebenfächer)

☐ **März, 2004 - Februar, 2005**

Diplomarbeit im Labor von Hubert Bahl an der Universität Rostock

Titel: Transkriptionsanalyse reverser Rubrerythingene aus *Clostridium acetobutylicum:* Stimuli und Operonstrukturen

☐ **März, 2005 bis Dezember, 2008**

Doktorand und wissenschaftlicher Mitarbeiter im Labor von Hubert Bahl in der Abteilung Mikrobiologie der Universität Rostock

☐ **September - November, 2006**

Erhalt eines „FEMS short term fellowship“

Titel: „Gene deletion in *C. acetobutylicum* in the laboratory of Philippe Soucaille at the Institute of Systems Biology“ am INSA Toulouse, Frankreich

☐ **April, 2008**

Transkriptomanalysen mit *C. acetobutylicum* im Labor von Armin Ehrenreich an der Universität Göttingen