Identification of O_2-induced peptides in an obligatory anaerobe, *Clostridium acetobutylicum*

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Abstract ***Clostridium acetobutylicum*** **DSM792 (= ATCC824), a solvent producing obligate anaerobe, grew well after a shift in growth conditions from anoxic to microoxic at the mid exponential phase. In two-dimensional gel electrophoresis, a spot migrating at 45 kDa and three spots at 23 kDa accumulated after 30 min of flushing with 5% O_2/95% N_2. Based on peptide mass fingerprints, the 45 kDa polypeptide was determined to be NP_347663 (A-type flavoprotein homologue) and the 23 kDa polypeptides were determined to be NP_350180 or NP_350181 (novel type rubrerythrin homologue). Northern blot analysis indicated that the expressions of these peptide transcripts were upregulated within 10 min after flushing with 5% O_2/95% N_2.**

Keywords: Obligate Anaerobe; Rubrerythrin; Flavoprotein; Oxygen-induced peptide; *Clostridium acetobutylicum*

1. Introduction

Although obligate anaerobes suffer aerobic growth inhibition, it is known that several anaerobic bacteria show oxygen tolerance and possess systems to respond to O_2. For example, sulfate reducing bacteria and *Bacteroides* species survive when exposed to oxygen by using systems that metabolize oxygen and active oxygen species [1–4].

The clostridia are typical obligate anaerobes [5,6] and widely used in investigations in the fields of solvent production, intestinal microflora, and microbial toxin production. Oxygen has a crucial effect on the growth of *Clostridium* [7–9]; however, the mechanism of growth inhibition as well as the existence of a molecular system to respond to O_2 remains unclear [10]. One popular explanation for aerobic growth inhibition is a lack of active oxygen scavenging systems such as catalase and SOD, but oxygen metabolic enzyme and active oxygen scavenging enzyme activities, such as those of NAD(P)H oxidase, NAD(P)H peroxidase, and SOD, are widely distributed in the crude extract of *Clostridium* species [11–13]. In addition,we have shown that *Clostridium aminovalericum*, an obligatory anaerobe, possesses an O_2-response H_2O-forming NADH oxidase that is capable of detoxifying oxygen to water [14]. The above results suggest that *Clostridium* species possess a system to metabolize or respond to oxygen.

In order to investigate the mechanism of O_2 response systems in Clostridia, we chose *Clostridium acetobutylicum*, a well-investigated strain whose entire genome has been sequenced and characterized [15]. It has been reported that *C. acetobutylicum* ceases growth under conditions of full aeration [11]. As in the case of *C. aminovalericum* [14], *C. acetobutylicum* grows well under microoxic conditions (flushing the medium with 5% O_2/95% N_2) without growth inhibition. Four polypeptides were detected as O_2-induced polypeptides by two-dimensional (2D) gel electrophoresis, and these were determined to be a novel type of rubrerythrin homologue and an A-type flavoprotein family homologue.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Clostridium acetobutylicum DSM792 (= ATCC824) was used in this study. *C. acetobutylicum* cells were grown in CGM [16] containing 2% (w/v) glucose, 0.5% yeast extract, 0.2% $(NH_4)_2SO_4$, 0.2% asparagine monohydrate, 0.1% NaCl, 0.075% K_2HPO_4, 0.075% KH_2PO_4, 0.075% $MgSO_4$, 0.01% $MnSO_4$, and 0.01% $FeSO_4$ at 37 °C (pH 7.0). Anoxic conditions were achieved by flushing the medium with O_2-free nitrogen gas in a jar-fermenter (10 liter jar-fermenter, model MDL1000, Marubishi-Bioenge, Tokyo, Japan) as described previously [14]. Aeration was achieved by flushing with air or with 5% O_2/95% N_2 mixed gas (a gas flow meter was used to fix the ratio of each gas) into the anaerobically growing strain at the mid-exponential phase ($OD_{660} = 1.5$). O_2-free N_2 gas or the aeration gas was sparged at a rate of 2 l/min, with an agitation rate of 60 rpm/min. Under these experimental conditions, the O_2-dissolving rate into the media was calculated to be 30–40 μM O_2/min when 5% O_2/95% N_2 was sparged. Oxygen concentrations in the media were measured with an oxygen electrode (O_2 monitor model 5331, Yellow Springs Instruments Co., Yellow Springs, OH, USA).

2.2. Protein extraction and two-dimensional electrophoresis

Peptide solutions were prepared and two-dimensional electrophoresis was performed as described previously with slight modification for bacterial peptide extraction [17]. Briefly, *C. acetobutylicum* cells were harvested before and after aeration and promptly frozen in liquid nitrogen. Proteins were sedimented out by acetone extraction, and the pellet was dried in vacuo and dissolved in 2D electrophoresis sample buffer containing 8 M urea, 30 mM DTT, 2% (v/v) Pharmalyte 3-10 (Pharmacia, Japan) and 0.5% Triton X-100. Both isoelectrofocusing (IEF) and SDS–polyacrylamide gel electrophoresis (SDS–PAGE) were performed horizontally with Maltiphor II (Pharmacia, Japan) according to the manual from Pharmacia. For electrophoresis, 10 μg of total protein was loaded onto gels. For detection, the gels were silver-stained with a SilverQuest™ Silver Staining Kit (Invitrogen, CA, USA).

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Abbreviation: 2D; Two-dimensional

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2.3. Spot excision and peptide mass fingerprinting

Peptide mass fingerprinting was performed according to the manufacturer's instructions (Shimadzu, Tsukuba, Japan). The peptide spots were excised with a scalpel and cut into pieces (1 mm × 1 mm). The spots were destained, and the following procedures for trypsin digestion and sample preparation for mass spectrometry analysis were performed according to the instruction manual (SilverQuest™ Silver Staining Kit, Invitrogen). Following destaining and trypsin digestion, the peptides were purified using a ZipTip CIS (Millipore, Bedford, Mass.) according to the manufacturer's instructions. A purified peptide solution was prepared with equal volumes of saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile–0.1% trifluoroacetic acid to create a sample template for matrix-assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS) (AXIMA-CFRplus; Shimadzu, Tsukuba, Japan). Peptide mass fingerprints were analyzed using Mascot software (Matrix Science, Ltd., London, UK).

2.4. Northern hybridization

Total RNAs from cell lysates harvested at several time points of aeration were isolated using TRIzol® (Invitrogen) according to the instruction manual. Chromosomal DNA was prepared and Northern blotting was carried out by standard procedures [18]. The RNA (15 μg) was electrophoresed in 1.0% agarose gels and blotted onto nylon membranes (Hybond N^+, Amersham, Japan). The membranes were probed with the entire coding sequence for CAC1027, or for CAC3597 and CAC3598. The CAC1027 gene was amplified by the polymerase chain reaction (PCR) with oligonucleotide primers 5′-ATGAGTG-CTGAAAAGCTTTGT′ and 5′-TAAACCTATAAAATCATCTAC-3′ using chromosomal DNA from *C. acetobutylicum* as a template. The genomic region containing both CAC3597 and CAC3598 was amplified by PCR with oligonucleotide primers 5′-ATACAGGAGAGGA-TGCTCCAG-3′ and 5′-AAACCTCTGAAAGCAGAACC-3′ using chromosomal DNA from *C. acetobutylicum* as a template. After the RNA was subjected to pre-hybridization at 60 °C for 30 min, the ^{32}P-labeled probe DNA was hybridized to RNA on the membrane at 60 °C for 12 h.

3. Results and discussion

3.1. Effect of O_2 on the growth of C. acetobutylicum

C. acetobutylicum, an obligatory anaerobe, develops colonies on the surface of PYD agar-plates in an atmosphere of 100% N_2, but not in a 1% O_2–99% N_2 atmosphere. When the strain is growing anaerobically in a jar-fermenter, it continues growing after a shift in the carrier-gas from O_2-free N_2 gas to 5% O_2/95% N_2 gas at the mid-exponential phase (OD_{660} = 1.5, Fig. 1). In these experiments, the concentration of O_2 dissolved in the culture medium remained at 0–0.2% until late stationary phase (Fig. 1). When 5% O_2/95% N_2 gas was sparged during the early exponential phase (OD_{660} = 0.3–0.4), the dissolved O_2 concentration in the culture medium increased quickly and cell growth stopped (data not shown). These findings suggest that *C. acetobutylicum* has an ability to grow microaerobically without growth inhibition if the O_2-dissolving rate is lower than the activity of O_2 reduction by the strain. These growth profiles closely resemble those of *C. aminovalericum*, as shown in our previous study [14].

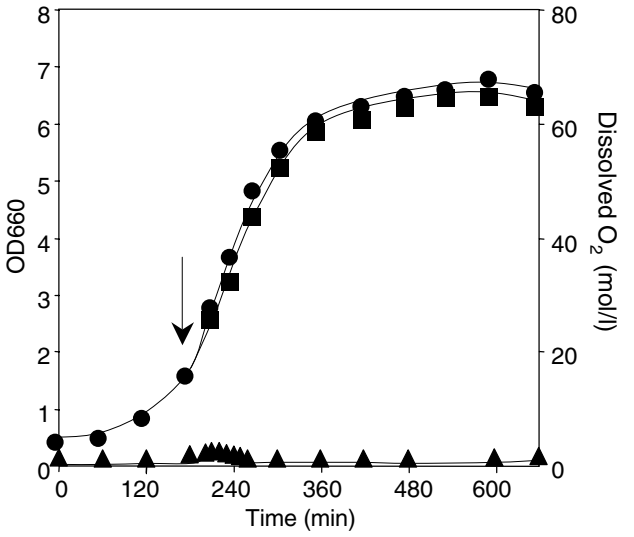

Fig. 1. Growth of *Clostridium acetobutylicum* under anoxic and microoxic conditions. Anaerobically precultured *C. acetobutylicum* cells were inoculated into a jar-fermenter. 5% O_2/95% N_2 gas (squares) was sparged at the mid exponential phase (arrow). Anoxic culture was monitored (circles). Triangles indicate dissolved O_2 concentrations (μmol O_2/liter medium) in the medium when the strain was cultured under 5% O_2/95% N_2 gas.

3.2. Analysis of oxygen-induced peptides

The above results suggest that *C. acetobutylicum* has systems to resist oxidative stress during aeration. We analyzed the changes in polypeptide expression after aeration by 2D electrophoresis. After 30 min of aeration, four polypeptides newly appeared and were named AR1–AR4 (Fig. 2). Molecular

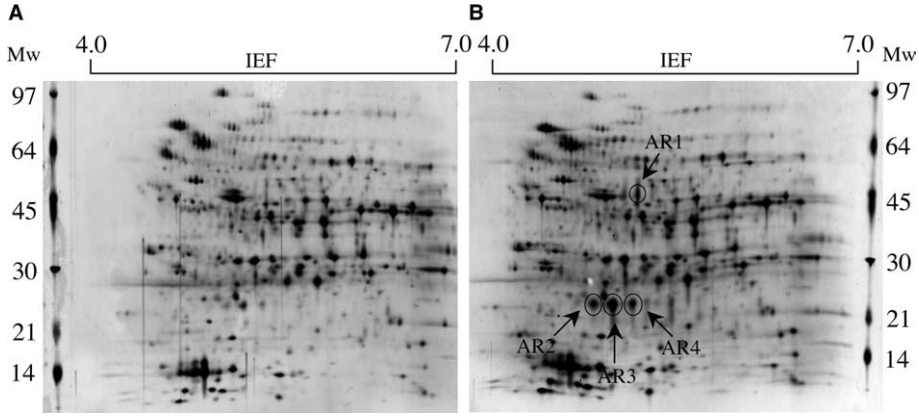

Fig. 2. Two-dimensional electrophoresis of polypeptides extracted from *C. acetobutylicum* cells. (A) Just before the start of aeration with 5% O_2/95% N_2 at the mid-exponential phase; (B) after 30 min of 5% O_2/95% N_2 aeration. Induced polypeptides are indicated by arrows and named AR1–AR4. Mw, molecular weight; IEF, isoelectric focusing.

weightS (M_w) and p*I* of AR1 are estimated to be 45 kDa and 5.3, respectively. AR2–AR4 show similar M_w (23 kDa) and p*I* values (5.0–5.3) to one another.

These peptide spots were identified by tryptic digestion of the excised spots from silver-stained gels, followed by MALDI-TOF MS analysis of the resulting digest mixture. The proteins identified were assigned appropriate CAC gene numbers by reference to the *C. acetobutylicum* strain ATCC 824 genome [15]. AR1 was determined to be NP_347663 (named flavoprotein in the genome project for *C. acetobutylicum*); AR2, AR3 and AR4 were determined to be NP_350180 or NP_350181, and were named rubrerythrin in the genome project for *C. acetobutylicum*. The functions of these peptides have not been characterized in *C. acetobutylicum*.

3.3. Genome structure and homology search

AR1 (Accession No. NP_347663, named flavoprotein) is encoded by CAC1027. The genome structure of CAC1027 is shown in Fig. 3. By database search of AR1 using the blastp program, highly homologous proteins were found in *C. tetani* (Accession No. NP_781654, named flavodoxin, 60% identity), *C. perfringens* (Accession No. NP_561930, named probable flavoprotein, 42% identity), and *Chlorobium tepidum* (Accession No. NP_663156, named putative rubredoxin:oxygen oxidoreductase, 39% identity). These highly homologous proteins identified by the genome project have not been characterized in terms of function. In the *C. acetobutylicum* genome, a paralog of AR1 is encoded by CAC2449 (Accession No. NP_349063, 41% identity), which is found at a different position on the genome, but its function is unknown. AR1 shows homology to a family of A-type flavoproteins and conserves the signature flavodoxin sequence ([LIV]–[LIVFY]–[FY]–X–[ST]–XX–[AG]–X–T–XXX–A–XX–[LIV]) [19]. A family of A-type flavoproteins is widely distributed in bacteria [19], and a few proteins in this family have been purified and characterized. In *Desulfovibrio gigas*, a sulfur reducing bacterium, FprA (Accession No. Q9FOJ6), is characterized as rubredoxin–oxygen oxidoreductase, which is suggested to be the final component in the electron transfer chain coupled with NADH oxidation [20]. On the other hand, *Moorella thermoacetica* FprA (Accession No. Q9FDN7) has been characterized and reported to show NADH:FprA oxidoreductase activity with the Hrp protein (high molecular weight rubredoxin, Accession No. Q9FDN6), the gene for which is co-transcribed with *FprA* [21]. Although *D. gigas* FprA and *M. thermoacetica* FprA show 27.8% and 31.9% identity to *C. acetobutylicum* AR1 (NP _349063), respectively, the numbers and the positions of conserved cysteine residues, which are important for iron-binding and oxidation-reduction reactions, do not correspond. *D. gigas* FprA, *M. thermoacetica* FprA, and *C. acetobutylicum* AR1 contain seven, four, and six conserved cysteine residues in each polypeptide, respectively, but these positions do not coincide with one another (data not shown). These results suggest that *D. gigas* FprA, *M. thermoacetica* FprA, and *C. acetobutylicum* AR1 belong to the same family of enzymes, but that their functional properties may not be the same.

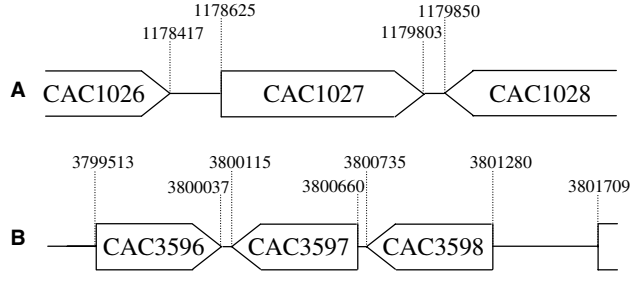

Fig. 3. Gene clusters around the genes encoding O_2-induced polypeptides. (A) CAC1027 locus, (B) CAC3597 and CAC3598 loci. Arrows indicate the direction of transcription. Positions on *C. acetobutylicum* chromosomal DNA (given above the diagrams) correspond to the assigned numbers given in the *C. acetobutylicum* genome project (Genbank Accession No. NC_003030)[15].

AR2–AR4 (Accession Nos. NP_350180 or NP_350181, named rubrerythrin) are encoded by CAC3597 or CAC3598. CAC3597 and CAC3598 are located in a same polycistronic unit (Fig. 3). At the nucleic acid level, both NP_350180 and NP_350181 show 98.9% identity to each other (6 nucleic acids difference in a 546 nt sequence), and the translated sequences show only one amino acid residue difference (no. 50 isoleucine in NP_350180 is valine in NP_350181). The theoretical p*I* values of NP_350180 and NP_350181 are both 5.40, suggesting that the different p*I* values of the three spots appearing on the gel are the result of post-translational modifications and not based on the one amino acid difference. A database search using the blastp program revealed highly homologous proteins in *C. perfringens* (Accession No. NP_563534, named conserved hypothetical protein, 79% identity), *C. tetani* (Accession No. NP_781487, named a protein with a rubredoxin/rubrerythrin domain, 78% identity), and *Bacteroides thetaiotaomicron* (Accession No. NP_809129, named as a putative rubrerythrin, 71% identity). None of these proteins identified in the genome project have been characterized in terms of function.

AR2–AR4 show low homology with *C. perfringens* rubrerythrin (Accession No. P51591, Rbr, encoded by *rbr*; 14% identity and 55% similarity to *C. acetobutylicum*; Fig. 3), whose function is suggested to be that of an SOD-like enzyme [22]. *C. perfringens* Rbr contains a non-sulfur iron-binding site, –Cys–X–X–Cys–$(X)_{12}$–Cys–X–X–Cys– [22]. This motif is located in the C-terminal region in *C. perfringens* Rbr, but in the N-terminal region in *C. acetobutylicum* NP_350180 and NP_350181 (Fig. 4). The C-terminal type rubrerythrins are well investigated and distributed in many anaerobic bacteria (*Desolfovibrio vulgaris* Accession No. P24931, *Porphyromonas gingivalis* Accession No. Q9AGG3, and *Moorella thermoaceticum* Accession No. AAF08979); their function has been proposed to be that of an SOD or cytoplasmic peroxidase [23–25]. The function of N-terminal type rubrerythrins has not been reported. A protein showing homology to a C-terminal type rubrerythrin has been found in the *C. acetobutylicum* genome (Accession No. NP_349184. encoded by CAC2575, 66% identity with *C. perfringens* Rbr).

3.4. Northern blot analysis

To investigate whether the expression of these proteins is regulated by oxygen at the transcriptional level, northern hybridization analysis was performed. The results clearly demonstrated that the mRNA encoding these proteins had been induced by 10 min after the start of aeration (Fig. 5). Northern

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Cace Rub       MKKFK**CVVCGYIYTGEDAPEKCPVC**GAGKDKFVEVKDEGEGWADEHKIGIAKG---VDKE   57
Cper Rbr       MKSLKGTKTAENLM-KSFAGECQARTRYTYFSSTARKEGYVQISNIFLETAENEKEHAKR   59
               **.:* .  .     :. . :* .    .     .:.**    .:  :  *:.     *.

Cace Rub       VLEGLRANFTGECTEVGMYLAMARQADREGYPEVAEAYKRIAFEEAEHASKFAELLGEVV  117
Cper Rbr       FYKFLKDDLQGEAVEINAAYPVELPTDTLTNLKFAAEGEHDELSNLYPS--FADVADEEG  117
               . : *: :: **..*:.   .:   :*     :.*   ::  :.:   :  **:: .*

Cace Rub       VADTKTNLQMRVDAEKGACEGKKELATLAKKLNYDAIHDTVHEMCKDEARHGSAFRG---  174
Cper Rbr       FPEVAAAFRMIAKAETAHYNRFMKLAKNMEEGKVFKKDEVVLWK**CGNCGFIWEGAEAPLK**  177
               ..:. : ::* ..**..  :   :**.  :: :    .:.*   * : .   .. ..

Cace Rub       --------LLNRYFK---                                            181
Cper Rbr       **CPAC**LHPQAFFEVFKETY                                        195
                       : . **
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Fig. 4. Alignment of *C. perfringens* rubrerythrin, whose function is known (Cper Rbr), and the *C. acetobutylicum* O_2-induced rubrerythrin homologue (NP_350180, Cace Rub). Identical amino acid residues are indicated by asterisks and similar amino acid residues are indicated by dots. The CXXC–$(X)_{12}$–CXXC clusters are written in boldface.

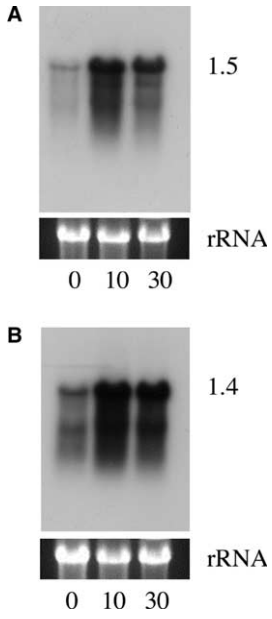

Fig. 5. Northern blot analysis of *C. acetobutylicum* total RNA probed with (A) the CAC1027 gene or (B) the CAC3597 and CAC3598 genes amplified by PCR. 0, just before the start of aeration with 5% O_2/95% N_2 at the mid-exponential phase; 10, after 10 min of 5% O_2/95% N_2 aeration; 30, after 30 min of 5% O_2/95% N_2 aeration. Estimated transcript sizes (in kilobases) are shown to the right of each blot. Ethidium bromide staining of the ribosomal RNA for confirmation of equal loading of RNA samples (rRNA) is shown below the autoradiograms.

blot analysis indicated that the CAC1027 transcript is monocistronic, and that CAC3597 and CAC3598 are both dicistronically transcribed.

3.5. Conclusions

In this study, we show that *C. acetobutylicum* grows well under microoxic conditions with the induction of O_2-response polypeptides. To our knowledge, this is the first report concerning *C. acetobutylicum* microoxic growth as well as the existence of O_2-induced polypeptides in Clostridia that are clearly regulated at the transcriptional level. The O_2-induced polypeptides were identified as an A-type flavoprotein protein and rubrerythrin homologues, suggesting that they may be involved in oxygen metabolism or in an active oxygen scavenging system. Further investigation of these peptides and the structure of the promoter region will help to clarify the role of these peptides and the molecular systems to respond to O_2.

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