A novel A3 group aconitase tolerates oxidation and nitric oxide

Yuki Doi and Naoki Takaya

From the Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan

**Background:** Aconitases are labile cellular targets of oxidative and nitrosative stresses.

**Results:** Aconitase A3 is resistant to and mitigates impaired growth and NADH and ATP generation by reactive nitrogen stress.

**Conclusion:** Aconitase A3 constitutes a novel group of bacterial aconitases for oxidation and reactive nitrogen tolerance.

**Significance:** The ecology and pathology of bacterial NO response are impacted.

*Achromobacter denitrificans* YD35 is an NO$_2^-$-tolerant bacterium that expresses the aconitase genes *acnA3*, *acnA4*, and *acnB*, of which *acnA3* is essential for growth tolerance against 100 mM NO$_2^-$. Atmospheric oxygen inactivated AcnA3 at a rate of 1.6 × 10$^{-3}$ min$^{-1}$, which was 2.7- and 37-fold lower compared with AcnA4 and AcnB, respectively. Stoichiometric titration showed that the [4Fe-4S]$^{2+}$ cluster of AcnA3 was more stable against oxidative inactivation by ferricyanide than that of AcnA4. Aconitase activity of AcnA3 persisted against high NO$_2^-$ levels that generate reactive nitrogen species with an inactivation rate constant of $k = 7.8 \times 10^{-3}$ min$^{-1}$, which was 1.6- and 7.8-fold lower than those for AcnA4 and AcnB, respectively. When exposed to NO$_2^-$, the *acnA3* mutant (AcnA3Tn) accumulated higher levels of cellular citrate compared with the other aconitase mutants, indicating that AcnA3 is a major producer of cellular aconitase activity. The extreme resistance of AcnA3 against oxidation and reactive nitrogen species apparently contributes to bacterial NO$_2^-$ tolerance. AcnA3Tn accumulated less cellular NADH and ATP compared with YD35 under our culture conditions. The accumulation of more NO by AcnA3Tn suggested that NADH-dependent enzymes detoxify NO for survival in a high NO$_2^-$ milieu. This novel aconitase is distributed in Alcaligenaceae bacteria, including pathogens and denitrifiers, and it appears to contribute to a novel NO$_2^-$ tolerance mechanism in this strain.

Aconitase (aconitase hydratase, EC 4.2.1.3) is an enzyme that contains an Fe-S cluster and converts citrate to isocitrate via cis-aconitate. The reaction is ubiquitous from bacteria to higher eukaryotes and constitutes an indispensable step in TCA cycle metabolism (1). Like other Fe-S-containing enzymes, aconitases are easily inactivated under oxidative conditions through disruption of their Fe-S clusters (2–4). Such inactivation impairs TCA cycle flux and energy conservation and hence cell proliferation. Bacterial aconitases are classified as aconitases A (AcnA) and B (AcnB) (5). *Escherichia coli* AcnA is induced by oxidative stress, and its activity resists oxidants in vitro compared with the other housekeeping isozyme, AcnB (5, 6). *Salmonella enterica* also produces these aconitase isozymes (7). *E. coli* aconitase C is identical to methylcitrate dehydratase (PrpD), which is involved in propionate catabolism and is also found in other propionate-utilizing bacteria (8–11). Other aconitase-related proteins have been identified, but their physiological functions and persistence against oxidative stress remain unknown.

Aerobic organisms can be exposed to stress caused by reactive oxygen species generated as byproducts of electron transport chains and by exogenously added stressors. Bacterial aconitases, including the relatively oxidation-tolerant AcnA isoforms, are among the most labile cellular targets of reactive oxygen species (3, 4, 6). Reactive nitrogen species (RNS)$^2$ are generated both in the environment and in endogenous bacterial cells, and they also damage aconitases and other enzymes containing Fe-S (12, 13). Nitric oxide (NO) is an RNS that induces oxidative stress in cells and causes various types of cytotoxicity, such as DNA denaturation, lipid oxidation, and enzyme inactivation (14). The innate immune system of mammals uses NO to kill infecting bacteria (15, 16). To accomplish infection, bacteria produce various tolerance mechanisms against attack by NO; flavohemoglobin, flavorubredoxin, and peroxiredoxin are NO-detoxifying enzymes that constitute bacterial NO tolerance mechanisms (17–19). The global nitrogen cycle generates RNS at high levels when the processes of denitrification and nitrification to nitrate are incomplete (20, 21). Thus, understanding the relationship between bacterial physiology and RNS is important.

Microorganisms can use nitrite (NO$_2^-$) both as a nitrogen source and as an electron acceptor for anaerobic respiration (20, 21). When protonated under acidic conditions, NO$_2^-$ generates RNS (22, 23). To determine bacterial responses to RNS, we isolated *Achromobacter denitrificans* YD35 from a water treatment tank as a strain that thrives in the presence of NO$_2^-$ concentrations as high 100 mM, and we investigated its RNS tolerance mechanisms (24, 25). Genetic screening of NO$_2^-$-hypersensitive mutants derived from YD35 with transposon insertions identified predicted genes involved in bacterial RNS tolerance. An example is the pyruvate dehydrogenase gene,
which provides acetyl-CoA, increases turnover of the TCA cycle, and hence generates NADH, which could be a substrate for enzymes that detoxify NO (25). This constitutes a possible RNS tolerance mechanism of YD35 and implies that the TCA cycle is important for bacterial adaptation to RNS.

Here, we investigated a strain that is hypersensitive to NO\textsubscript{2}\textsuperscript{-} and harbors a mutation at the predicted aconitase gene (acrA3). We found that AcnA3 is important for TCA cycle metabolism by isomerizing citrate in the presence of high NO\textsubscript{2}\textsuperscript{-} concentrations. The aconitase activity of AcnA3 was highly resistant to oxidation and NO in vitro compared with other aconitase isozymes from this bacterium. *A. denitrificans* AcnA3 and its counterparts from the closely related *Bordetella* constitute a clade with the distinct AcnA of the Alcaligenaceae family of bacteria, and they restored the NO\textsubscript{2}\textsuperscript{-} sensitive growth of the acnA3 mutant. These findings show that AcnA3 constitutes a novel group of aconitases that tolerate oxidation and extreme high RNS, and it enables oxidation-tolerant TCA cycle metabolism in bacteria.

**EXPERIMENTAL PROCEDURES**

**Strains, Culture, and Media—Supplemental Table 1** shows the bacterial strains and plasmids used in this study, and they were cultured and propagated in DM (0.5% Polypeptone, 0.3% nutrient broth, 0.4% NaCl, 0.1% K\textsubscript{2}HPO\textsubscript{4}, and 0.2% trace element solution (25), pH 7.0), LB (1% Tryptone, 0.5% yeast extract, and 0.5% NaCl), YEB (0.75% yeast extract and 0.8% nutrient broth), and Super Optimal broth with catabolite repression (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl\textsubscript{2}, 6H\textsubscript{2}O, 10 mM MgSO\textsubscript{4}, 7H\textsubscript{2}O, and 20 mM glucose, pH 7.0) media. Bacterial strains were cultured at 120 rpm and 30 °C under aerobic conditions.

**Gene Disruption of *A. denitrificans* Genes**—The tetracycline resistance gene (tet) from pBSL199 was amplified by PCR using KOD-Plus DNA polymerase (Toyobo Co., Osaka, Japan) and primers (supplemental Table 3), digested with EcoRI, and cloned into the same restriction site of pAcnA4 and pAcnB (see below for construction) to generate pAcnB::tet and pAcnA4::tet, which were digested with XbaI and HindIII. The *acnA4::tet* gene fragment was cloned into pHSG396 (Takara Bio Inc., Shiga, Japan) to generate pAcnA4\textsubscript{KO}. After digesting pAcnB::tet with XbaI and SacI, the *acnB::tet* gene fragment was cloned into pHSG396 to generate pAcnB\textsubscript{KO}. These plasmids transformed *A. denitrificans* YD35 by electroporation (25), and transformants were cultured on YEB plates containing 20 mg/liter tetracycline sulfate. Gene disruptions were confirmed by PCR, and we designated the strains AcnA4\textsubscript{Δ} and AcnB\textsubscript{Δ} (Fig. 1).

**Preparation of Recombinant Aconitase**—Total DNA from bacterial strains was purified using DNeasy blood and tissue kits (Qiagen, Hilden, Germany). *acnA3* (accession number LC003604), *acnA4* (LC003605), and *acnB* (LC003606) were amplified by PCR using a GC-rich PCR system (Roche Diagnostics), gene-specific primers (supplemental Table 3), and total DNA as a template, were cloned into pET28a (Novagen, Madison, WI). AcnA3, AcnA4, and AcnB were produced using plasmids pAcnA3, pAcnA4, and pAcnB, respectively. Recombinant aconitases were prepared and purified as described (25).

**Aconitase Assays**—Aconitase activity was determined as decreasing absorption at 240 nm derived from cis-aconitate. Typical reactions contained aconitate (0.1–1 μg), 0.2 mM cis-aconitate, and 50 mM Tris-HCl (pH 8.0) and were incubated at 25 °C. The molar extinction coefficient of cis-aconitate (ε\textsubscript{240} = 3.41 M\textsuperscript{-1} cm\textsuperscript{-1}) was used to calculate aconitase activity (26). Purified aconitases were reactivated by incubation with 1 mM (NH\textsubscript{4})\textsubscript{2}Fe(SO\textsubscript{4})\textsubscript{2} and 5 mM dithiothreitol in 50 mM Tris-HCl (pH 7.0) on ice for 10 min.

**Reverse Transcription and Real-time PCR**—Total RNA was purified using RNeasy Protect bacteria mini kits (Qiagen), and cDNA was synthesized using QuantiTect reverse transcription kit (Qiagen). Specific amplicons for the 16 S rRNA, *acnA1*, *acnA2*, *acnA3*, *acnA4*, and *acnB* genes were amplified by PCR using cDNA, KOD-Plus DNA polymerase, and primers (supplemental Table 3). Real-time PCR was carried out using SYBR Green Supermix and MiniOpticon version 3.1 (Bio-Rad) with the primers (supplemental Table 3) recommended by the manufacturer. The amounts of generated transcripts are shown relative to those of the 16 S rRNA gene.

**Production of Aconitases in the AcnA3Tn Strain**—We prepared plasmids to introduce aconitase genes into AcnA3Tn by amplifying aconitase gene fragments using PCR and the primer sets used to prepare recombinant aconitases. The PCR products were digested with Nhel and HindIII and cloned into Nhel/HindIII-digested pNSGroE2 to generate pNS-xxx (where xxx denotes the protein name of the aconitase produced). Transformants harboring the plasmid were selected and maintained in DM containing 50–500 mg/liter chloramphenicol.
Aconitase A3 Tolerates Nitric Oxide

Preparation of AcnA3 Mutant Proteins—The 5’-regions of the acnA3 genes were amplified using primer AcnA3_PF and primer C437S_R, C503S_R, or C506S_R. The 3’-regions of the acnA3 genes were amplified using primer AcnA3_PR and primer C437S_F, C503S_F, or C506S_F. The respective amplified DNA fragments were fused by PCR using primers AcnA3_PF and AcnA3_PR, digested with PstI and SalI, and cloned into PstI and SalI-digested pAcnA3. Mutant proteins were then expressed in E. coli using the resulting pAcnA3_C437S, pAcnA3_C503S, and pAcnA3_C506S. Supplemental Table 3 shows the primers used in these processes.

NO Determination—Strains were cultured in DM with 1 mM diaminohodamine-4M acetoxyethyl ester (Sekisui Medical Co., Ltd., Tokyo, Japan). After culture for 12 h, 100 mM NO<sub>2</sub> was added, and cultures were continued for 12 h. The cells were collected by centrifugation, washed twice with 20 mM sodium phosphate (pH 7.0) containing 0.8% NaCl, and resuspended at pAcnA3, and pAcnA3_C506S. Cellular citrate was extracted from cells using cold methanol as described (27) and analyzed using a Roche Yellow line kit (R-Biopharm AG, Darmstadt, Germany) or a GC/MS-QP2010 Plus gas chromatograph-mass spectrometer (Shimadzu Co., Kyoto, Japan). Proteins were resolved by SDS-PAGE according to Laemmli (28).

RESULTS

Identification of a Novel acnA3 Gene That Tolerates NO—We isolated mutants with NO<sub>2</sub>-hypersensitive growth by transposon mutagenesis of A. denitrificans YD35 and identified four mutants with transposon insertions at acnA3 loci (Fig. 2A) (25). One of the mutants (AcnA3Tn) did not grow in the presence of >60 mM NO<sub>2</sub> and >3 mM acidified NO<sub>2</sub> (pH 5.5) (Fig. 2B). At low pH, NO<sub>2</sub> is protonated and generates RNS, including NO (22, 23), indicating that the growth of AcnA3Tn was sensitive to RNS generated from the acidified NO<sub>2</sub>. In addition to the single acnB gene, the YD35 strain genome encoded four potential acnA genes, which we termed acnA1, acnA2, acnA3, and acnA4 according to the designations of the corresponding Bordetella avium 197N genes (accession number AM167904). RT-PCR showed that only acnA3, acnA4, and acnB were expressed in the presence or absence of added NO<sub>2</sub> under our culture conditions (Fig. 2C). Quantitative PCR indicated that more acnB and acnA4 were transcribed during the early logarithmic growth phase (6 h), whereas more acnA3 was expressed during the late logarithmic growth phase (12–18 h) (Fig. 2D). The cellular aconitase activity of AcnA3Tn was 30% of that of the YD35 strain during the early logarithmic growth phase, and it decreased to <10% during the late logarithmic growth phase (Fig. 2E), indicating that AcnA3 was produced throughout the culture period and was expressed predominantly during the late logarithmic growth phase.

AcnA3 Is a Unique NO-tolerant Aconitase Isozyme—We constructed gene disruptants of acnA4 (AcnA4Δ) and acnB (AcnBΔ) (Fig. 1). When cultured in the absence of NO<sub>2</sub>, AcnA4Δ, AcnBΔ, and AcnA3Tn proliferated at the same rate as
Aconitase A3 Tolerates Nitric Oxide

JANUARY 16, 2015 • VOLUME 290 • NUMBER 3

A coni A3 Tolerates Nitric Oxide

YD35 (Fig. 3A). Adding 100 mM NO₂⁻ to the medium partially inhibited the growth of the AcnA4Δ and AcnBΔ strains and that of the AcnA3Tn strain more obviously (Fig. 3B). The effect of acidified NO₂⁻ on AcnA4Δ and AcnBΔ growth was very small, but was more obvious with AcnA3Tn (Fig. 2B). We introduced acnA3, acnA4, and acnB into AcnA3Tn using a multicopy vector, and the resulting transformants produced 8–14-fold more aconitase activity in cell extracts compared with YD35 (Fig. 3C) due to the gene dosage effect. Producing AcnA4 did not restore the growth defect of AcnA3Tn in the presence of 100 mM NO₂⁻, whereas the effect of AcnB was partial, relative to the strain overproducing AcnA3 (Fig. 3D). These results indicate that AcnA3 is distinguishable from AcnA4 and AcnB by its role in bacterial NO-tolerant growth.

AcnA3 mutants in which the potential iron-binding Cys-437, Cys-503, and Cys-506 residues with replaced with Ser were produced by E. coli and did not bind to the Fe-S cluster (Fig. 4A). Their corresponding genes restored neither the defective cellular aconitase activity nor the NO₂⁻-sensitive growth of AcnA3Tn (Fig. 4, B and C). Aconitases without an Fe-S cluster regulate protein translation and control the translation of ferritin and other iron-related proteins (29). Although the function of AcnA3 as a translation regulator in A. denitrificans remains elusive, this confirms that AcnA3 tolerates NO independently from translation regulation. It rather tolerates NO through the TCA cycle mechanism that generates NADH, as discussed below.

We examined the stability of cellular aconitase activity upon exposure to NO₂⁻. When 100 mM NO₂⁻ was added to the culture medium at mid-logarithmic growth phase (t = 0) (Fig. 3E), AcnA3Tn proliferated at a lower rate compared with YD35, AcnA4Δ, and AcnBΔ, confirming that AcnA3 is important for NO₂⁻ tolerance. AcnA3Tn almost completely lost cellular aconitase activity after a 12-h exposure, whereas the other strains persisted at 30% of the activity (Fig. 3F). These results indicate that the aconitase activity of AcnA3 is more resistant to NO in vivo than that of AcnA4 and AcnB.

Distribution of AcnA3-type Aconitases among β-Proteobacteria—The existence of an RNS-resistant aconitase AcnA3 was intriguing because the activities of common aconitases are sensitive to oxidative stress caused by atmospheric oxygen and NO due to oxidation-labile Fe-S clusters. We therefore phylogenetically analyzed the novel aconitase isozyme to understand its distribution and classification. Bacterial aconitases consist of the AcnA and AcnB families (5, 30). The results of the phylogenetic analyses of AcnA family proteins indicated that the AcnA isozymes of α-, β-, γ-, and δ-proteobacteria were grouped into respective clades comprising each proteobacterium, whereas the AcnA of γ-proteobacteria resided in two separate clades (Fig. 5). These results suggest the emergence of a common ancestor before these proteobacteria diversified. The β-proteobacteria group included YD35 AcnA3 and its counterparts from related bacteria, indicating that AcnA3-like aconitases are distributed in β-proteobacteria. Potential methylcitrate dehydratase constitutes another clade containing mostly proteins of β- and γ-proteobacteria, and YD35 AcnA4 belongs to this clade, which reflects its potential function as methylcitrate dehydratase (Fig. 5). Some of the AcnA isoforms were not placed into any of these groups and were unrelated to any proteins with known function, and their physiological functions were not evident (Fig. 5). These isoforms included YD35 AcnA1 and AcnA2. To date, the catalytic properties,
Aconitase A3 Tolerates Nitric Oxide

Aconitase A3 Tolerates Nitric Oxide

Analysis of AcnA3 mutants. A, SDS-PAGE analysis (upper panel) and images (middle panel) of purified AcnA3, AcnA3C437S, AcnA3C437Sf, and AcnA3C503S, and absorption spectra of purified AcnA3 and AcnA3C437S (lower panel). Absorption spectra of AcnA3C503S and AcnA3C506S were indistinguishable from that of AcnA3C437S (data not shown). B, Acn activities of YD35 and AcnA3Tn harboring pNSGroE2 (none), pNS-AcnA3, pNS-AcnA3C437S, pNS-AcnA3C503S, and pNS-AcnA3C506S cultured in DM. Data are means of three experiments. Error bars indicate S.E. C, growth of YD35 (C) and AcnA3Tn harboring pNS-AcnA3 (●), pNS-AcnA3C437S (▲), pNS-AcnA3C503S (■), and pNS-AcnA3C506S (▲) in DM containing 100 mM NO2.

Mechanisms for NO Tolerance by AcnA3 in Vivo—One major aconitase function is citrate isomerization in TCA cycle metabolism. Strains lacking AcnA3, AcnA4, or AcnB accumulated more cellular citrate compared with YD35, indicating that they oxidation, and NO tolerance of β-proteobacteria-type aconitases, including YD35 AcnA3, remain largely unknown.

Oxygen/NO Tolerance of AcnA3 Activity—Oxidants damage the labile [4Fe-4S]2+ cluster to generate [3Fe-4S]1+ and inactivate aconitases (1, 31). We purified recombinant AcnA3, AcnA4, and YD35 AcnB produced in E. coli under aerobic conditions. AcnA3 and AcnA4 exhibited 107 and 27 μmol/min/mg aconitase activity, respectively (Table 1). Gel filtration chromatography showed that AcnA3 and AcnA4 were monomers with molecular weights of 78,000 and 97,000, respectively, and they were both active over a broad pH range (Fig. 6). Purified AcnB was less active, but when activated by incubation with dithiothreitol and ferrous ion (Fe2+), its activity reached 13 μmol/min/mg (Table 1). The activation procedures did not much affect AcnA3 and AcnA4. Taken together with the following spectroscopic findings, these results indicate that AcnA3 and AcnA4 are more stable against oxidative inactivation during purification compared with AcnB-like E. coli AcnA (6). Exposure to 20% oxygen revoked the activated AcnB and, to a lesser extent, AcnA3 and AcnA4. This inactivation of AcnA3, AcnA4, and AcnB followed pseudo first-order kinetics, with kinetic constants of 1.6 ± 0.4 × 10−3, 4.3 ± 0.1 × 10−3, and 5.8 ± 0.3 × 10−2 min−1, respectively (Fig. 7A), indicating that the AcnA3 activity was more stable against oxygen than the AcnA4 and AcnB activities. Visible spectrometry of AcnA3 revealed an absorption peak at 410 nm (Fig. 7B), which is typical of aconitases in the reduced [4Fe-4S]2+ state (31). Adding 10 μM ferricyanide increased absorption at 480 nm, indicating that the reduced [4Fe-4S]2+ was oxidized to the [3Fe-4S]1+ state, as reported previously (31). Adding dithionite reduced AcnA3, decreased absorption at 410 nm, and resulted in a spectrum characteristic of [4Fe-4S]1+ aconitases (31). On the other hand, the [4Fe-4S]2+ of AcnA4 was not reduced to the [4Fe-4S]1+ state by adding 10 μM dithionite. The absorption spectrum elicited by ferricyanide-dependent oxidation changed AcnA4 similarly to AcnA3, but the spectral changes against the ferricyanide were more sensitive than those of AcnA3. The apparent rate constant of AcnA4 oxidation by 10 μM ferricyanide was 1.9 ± 0.3 min−1, which was 10-fold higher than that of AcnA3 (0.19 ± 0.01 min−1) (Fig. 7C). These results indicate that the AcnA3 activity is resistant to oxidants.

A 4-h incubation with 100 mM NO2− decreased AcnA4 and AcnB activities to 13 and 1% of those before incubation, whereas 41% of the AcnA3 activity remained (Fig. 7D). The apparent kinetic constants for AcnA3, AcnA4, and AcnB inactivation were 7.8 ± 0.1 × 10−3, 1.3 ± 0.1 × 10−2, and 6.1 ± 0.1 × 10−2 min−1, respectively (Fig. 7D). This indicates that AcnA3 activity is more resistant to NO than the other aconitase isozymes and agrees with the contribution of AcnA3 to the NO-tolerant growth of YD35. The redox potential of the [4Fe-4S]2+ cluster of AcnA3 might be lower than that of AcnA4, but further electrochemical studies are required to confirm this speculation.
isomerize citrate in vivo (Fig. 8A). Less citrate accumulation in AcnBΔ than in the other aconitase mutants is in contrast to the major role of *E. coli* AcnB in citrate metabolism (5, 32). Incubating YD35 and the aconitase mutants with a high concentration of NO2− increased cellular citrate levels. The more obvious increase in the AcnA3Tn cells (Fig. 8A) indicated that AcnA3 played a major role in the cells incubated with NO2−. Incubation with high NO2− concentrations decreased the cellular NADH/NAD+ ratio (Fig. 8B) and the ATP concentration (Fig. 8C), which supports our concept that the up-regulated TCA cycle
Aconitase A3 Tolerates Nitric Oxide

**TABLE 1**

Specific activity of bacterial aconitases

| Strain                          | Accession number | Purified                      | Activated                      |
|---------------------------------|------------------|-------------------------------|-------------------------------|
|                                 |                  | μmol/min/mg                   |                               |
| AcnA family                     |                  |                               |                               |
| α-Proteobacteria                |                  |                               |                               |
| *O. anthropi* ATCC 49188<sup>T</sup> | Ocean_0105      | 36 ± 3                        | 33 ± 2                        |
| *S. japonicum* UT265            |                  | 30 ± 1                        | 26 ± 1                        |
| β-Proteobacteria                |                  |                               |                               |
| *A. denitrificans* YD35         | AcnA3            | 107 ± 3                       | 103 ± 4                       |
| *B. bronchiseptica* NBRC 13691<sup>T</sup> | BB1844          | 82 ± 3                        | 76 ± 4                        |
| *D. acidovorans* NBRC 14950<sup>T</sup> | Daci_2441      | 73 ± 9                        | 69 ± 10                       |
| *R. eutropha* NBRC 102504       | H16_A2638        | 74 ± 3                        | 67 ± 4                        |
| γ-Proteobacteria                |                  |                               |                               |
| *E. coli* DH10B                  |                  |                               |                               |
| *P. aeruginosa* PAO1             | AcnA4            | 27 ± 2                        | 25 ± 1                        |
| Methylcitrate dehydratase       |                  |                               |                               |
| *A. denitrificans* YD35         |                  |                               |                               |
| *D. acidovorans* NBRC 14950<sup>T</sup> | Daci_3587      | 25 ± 1                        | 26 ± 1                        |
| *P. aeruginosa* PAO1             | PA0794           | 36 ± 1                        | 31 ± 1                        |
| *R. eutropha* NBRC 102504       | H16_A1907        | 33 ± 3                        | 25 ± 1                        |
| AcnB family                     |                  |                               |                               |
| *A. denitrificans* YD35         | AcnB             | 0.6 ± 0.1                     | 13 ± 0.4                      |
| *E. coli* DH10B                  | ECDH10B_0098     | 1.4 ± 0.3                     | 3 ± 0.2                       |
| *P. aeruginosa* PAO1             | PA1787           | 1.6 ± 0.2                     | 11 ± 2                        |
| *R. eutropha* NBRC 102504       | H16_B0568        | 0.7 ± 0.1                     | 1.6 ± 0.1                     |

**FIGURE 6.** **Properties of purified AcnA3 and AcnA4.** A. dependence of aconitase activity upon pH. Reactions were buffered with 50 mM MES-HCl/NaOH (●), 50 mM MOPS-HCl/NaOH (▲), 50 mM Tris-HCl/NaOH (▽), and 50 mM CAPS-HCl/NaOH (●); 50 mM CHES-HCl/NaOH (◇), 50 mM Tris-HCl/NaOH (▲), and 50 mM CAPS-HCl/NaOH (●); and 50 mM CHES-HCl/NaOH (◇). B, purified recombinant AcnA3 and AcnA4 were analyzed by gel filtration chromatography using a Superose 6 10/300 GL column. Molecular weights were calibrated using thyroglobulin (669 kDa), ovalbumin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), and carbonic anhydrase (29 kDa); mAU, milli-absorbance units.

Increases cellular NADH and ATP and adapts the cells to oxidative stress caused by NO<sub>2</sub> (25). These results show that AcnA3 rendered the cells NO-tolerant via the TCA cycle. The NADH produced by the TCA cycle also served as a substrate for RNS-detoxifying enzymes, which are up-regulated by NO<sub>2</sub> (Fig. 8D) (25) and which decrease cellular NO levels, because we found that 6-fold more NO accumulated in AcnA3Tn than in YD35 (Fig. 8E). The growth of AcnA3Tn was insensitive to other oxidative stressors, such as hydrogen peroxide, r-butyl hydroperoxide, and methyl viologen, relative to the wild type (Fig. 8F). Menadione (3 mM) slightly affected the growth of AcnA3Tn and other mutants. These results indicate that the tolerance mechanism of AcnA3 is specific to NO and menadione among oxidants.

Distribution of Oxidation-tolerant Aconitases—Oxygen sensitivity was investigated by comparing the activities of aerobiologically purified recombinant α-, β-, and γ-proteobacterial sources of AcnA aconitases, four methylcitrate dehydratases, and four AcnB aconitases from various bacteria before and after activation by dithiothreitol and Fe<sup>2+</sup> (Table 1). The results showed that all of the AcnA aconitases, including methylcitrate dehydratases, were active irrespective of the type of activation and that all four AcnB aconitases required activation to generate maximal activity. These results indicate that AcnA activity is more oxygen-stable compared with AcnB activity, like the *E. coli* isozymes (6). In addition, the specific activity of the β-proteobacterial aconitases, including YD35 AcnA3 (Fig. 5), was higher than that of the aconitases from the other sources after activation (Table 1). This might account for their role in bacterial NO tolerance (see below).

The contribution of bacterial AcnA to NO tolerance was examined by replacing AcnA3 in YD35 cells with other aconitases. The aconitase activities of the strains producing AcnA isozymes from *Ochrobactrum anthophi* (Oant_0105), *Sphingobium japonicum* (SJA_C1-23180), *Bordetella bronchiseptica* (BB1844), *Delftia acidovorans* (Daci_2441), *Ralstonia eutropha* (Daci_3587), *Ochrobactrum anthropi* (ECDH10B_1393), and *Pseudomonas aeruginosa* (PA1562) were as high as or even higher than that of *Pseudomonas aeruginosa* (H16_A2683), *E. coli* (ECDH10B_1393), and *Ochrobactrum anthropi* (Oant_0105), *Sphingobium japonicum* (SJA_C1-23180), *Bordetella bronchiseptica* (BB1844), *Delftia acidovorans* (Daci_2441), *Ralstonia eutropha* (Daci_3587), *Ochrobactrum anthropi* (ECDH10B_1393), and *Pseudomonas aeruginosa* (PA1562) were as high as or even higher than that of YD35 (Fig. 9A), confirming the heterologous production of active enzymes. The production of AcnA from *R. eutropha* and *D. acidovorans* (class, β-proteobacteria; order, Burkholderiales; family, Ralstoniaceae and Comamonadaceae, respectively) did not recover the growth defect of AcnA3Tn (Fig. 9B). The growth deficiency was recovered by producing AcnA3 from YD35 (Fig. 3D) and *B. bronchiseptica* (Fig. 9B). These bacteria belong to the Alcaligenaceae family of β-proteobacteria. None of the AcnA isozymes from *O. anthropi* and *S. japonicum* (both...
α-proteobacteria) and E. coli and P. aeruginosa (both γ-proteobacteria) fully recovered the NO$_2^-$-tolerant growth of AcnA3Tn, but these effects were poorly reproducible. These results indicate that AcnA3 from bacteria only of the Alcaligenaceae family confers RNS-tolerant growth.

**DISCUSSION**

We screened A. denitrificans YD35 genes that tolerated high NO$_2^-$ levels and discovered the acnA3 gene. The enzyme activity of aconitases is often sensitive to oxidation by oxygen and reactive oxygen species (such as superoxide) both in vivo and in vitro (3, 4, 6). E. coli and most likely other bacteria produce the aconitase AcnA isozymes, which are more oxidation-resistant than AcnB and which maintain TCA cycle metabolism to facilitate growth in the presence of superoxide (5, 6, 32). However, the functions of bacterial aconitase in the presence of NO have rarely been characterized. A. denitrificans YD35 AcnA3 is a newly characterized aconitase A isozyme; its enzyme activity is more resistant to oxidants compared with other aconitase isozymes. Genetic studies indicated that the AcnA3 of this and related bacteria enables bacterial cells to grow in the presence of high NO$_2^-$ levels. This implies that AcnA3 is indeed an AcnA and that its function in the bacterial NO tolerance mechanism is unique.

The molecular mechanisms through which aconitases produce catalytic activity have been reported. E. coli AcnB produces activity through dimerization (33). However, we purified active YD35 AcnA3 as a monomer (Fig. 6B), indicating that dimerization is not required for the stability of AcnA3 against oxygen. The present spectrometric study showed that atmospheric oxygen inactivated AcnA3 at a lower rate compared with AcnA4 (Fig. 7A) and probably other AcnA and AcnB isozymes, suggesting that the redox potential of the Fe-S cluster is important for stability against oxygen. The overall amino acid sequence similarity between AcnA3 and other aconitases predicts that AcnA3 essentially shares a tertiary structure with other aconitases and consists of four domains (1). Domain 4 binds the fourth iron atom of the Fe-S cluster and regulates aconitase substrate (citrate) and ligand (mRNA) binding when functioning as an iron-responding protein (29, 34, 35). Amino acid substitutions in domain 4 and the adjacent linker region are likely to affect the redox potential and hence the oxygen and NO stability of AcnA3, through which the bacterium acquired NO tolerance. However, detailed studies are required to confirm this notion.

We identified 16 A. denitrificans genes that are involved in NO tolerance, and they are also involved in various cellular processes (25). Some of them notably encode pyruvate dehydrogenase, as well as α-ketoglutarate dehydrogenase and cytochrome bd-type terminal oxidase, which are both involved in energy conservation processes. Together with the role of
Aconitase A3 Tolerates Nitric Oxide

AcnA3 in generating ATP (Fig. 8), these observations suggest that ATP generation is critical for growth persistence in the presence of NO. Previous findings have also indicated that acetyl-CoA produced by pyruvate dehydrogenase is a key metabolite in tolerance to high levels of NO\textsubscript{2} (25). In such situations, up-regulated TCA cycle metabolism uses acetyl-CoA to generate more NADH, which is a substrate for NO-detoxifying enzymes. YD35 exposed to NO\textsubscript{2} up-regulated NO-detoxifying enzymes, such as flavohemoglobin and alkyl hydroperoxide reductase (Fig. 8D) (25). These enzymes require NADH to detoxify NO and RNS (36, 37). The higher levels of cellular NO in AcnA3Tn exposed to NO\textsubscript{2} compared with YD35 (Fig. 8E) suggested impaired NO degradation in AcnA3Tn. Residual cellular NO inactivated less NO-sensitive AcnB and AcnA4 (Figs. 3F and 7), which, in the absence of AcnA3, attenuated TCA cycle turnover (Fig. 8A) and caused a decrease in the NADH/NAD\textsuperscript{+} ratio (Fig. 8F). Thus, in addition to energy conservation, NO detoxification capacity depends on NADH generated by AcnA3, which enables efficient turnover of the TCA cycle under conditions of NO stress.

Bacterial AcnB was partially oxidized and inactivated during aerobic purification, which is in sharp contrast to the 12 tested AcnA isozymes in which enzyme activity persisted during the same type of purification (Table 1). When produced in A. denitrificans AcnA3Tn, these oxidation-resistant AcnA isozymes conferred various degrees of bacterial growth tolerance against NO. Only AcnA3Tn producing AcnA3 from B. bronchiseptica, which belongs to the same family as A. denitrificans (Alcaligenaceae), grew as much as the YD35 strain in the presence of high NO\textsubscript{2} levels (Fig. 9B). This indicates that the oxygen tolerance that afforded persistent enzyme activity during aerobic purification cannot account for the bacterial growth tolerance to NO. An additional mechanism must therefore participate in AcnA activity tolerance against NO.

The AcnA3-type oxidation/RNS-tolerant aconitases were distributed only in bacteria of the Alcaligenaceae family identified so far. This family includes the typical denitrifiers A. denitrificans and Achromobacter xylosoxidans, which conserve energy through the respiratory reduction of NO\textsubscript{2} as well as NO\textsubscript{2} (38). A. denitrificans YD35 was notably isolated from an aquarium water tank with a...
high level of NO$_2^-$ contamination, and it is a denitrifier (24, 25). The AcnA3 generated by this species of bacteria contributes to the ability of the bacteria to thrive in very high NO$_2^-$ concentrations, against which conventional bacteria have no resistance. The Alcaligenaceae family also contains the whooping cough pathogen *Bordetella pertussis* and other *Bordetella* subspecies (39). Infectious bacteria are considered to produce NO tolerance mechanisms for surviving NO generated by host phagocytes. Our findings suggest that AcnA3 isozymes also constitute a novel NO tolerance mechanism in these pathogens and that AcnA3 might serve as a novel target for developing medical treatments to cure and prevent disease.

Acknowledgment—We thank Norma Foster for critical reading of the manuscript.

REFERENCES

1. Beinert, H., Kennedy, M. C., and Stout, C. D. (1996) Aconitase as iron-sulfur protein, enzyme, and iron-regulatory protein. *Chem. Rev.* 96, 2335–2374
2. Flint, D. H., Tuminello, J. F., and Emptage, M. H. (1993) The inactivation of Fe-S cluster containing hydro-lyases by superoxide. *J. Biol. Chem.* 268, 22369–22376
3. Gardner, P. R., and Fridovich, I. (1991) Superoxide sensitivity of the *Escherichia coli* aconitase. *J. Biol. Chem.* 266, 19328–19333
4. Gardner, P. R., and Fridovich, I. (1992) Inactivation-reactivation of the aconitase in *Escherichia coli*. A sensitive measure of superoxide radical. *J. Biol. Chem.* 267, 8757–8763
5. Gruer, M. J., and Guest, J. R. (1994) Two genetically-distinct and differentially-regulated aconitases (AcnA and AcnB) in *Escherichia coli*. *Microbiology* 140, 2531–2541
6. Varghese, S., Tang, Y., and Imlay, J. A. (2003) Contrastings sensitivities of *Escherichia coli* aconitases A and B to oxidation and iron depletion. *J. Bacteriol.* 185, 221–230
7. Baothman, O. A., Rolle, M. D., and Green, J. (2013) Characterization of *Salmonella enterica* serovar Typhimurium aconitase A. *Microbiology* 159, 1209–1216
8. Blank, L., Green, J., and Guest, J. R. (2002) AcnC of *Escherichia coli* is a 2-methylcitrate dehydratase (PrpD) that can use citrate and isocitrate as substrates. *Microbiology* 148, 133–146
9. Brock, M., Maeker, C., Schütz, A., Völker, U., and Buckel, W. (2002) Oxidation of propionate to pyruvate in *Escherichia coli*. Involvement of methylmalonyl dehydratase and aconitase. *Eur. J. Biochem.* 269, 6184–6194
10. Horswill, A. R., and Escalante-Semerena, J. C. (1999) *Salmonella typhimurium* LT2 catalyzes propionate via the 2-methylcitrate cycle. *J. Bacteriol.* 181, 5615–5623
11. Claes, W. A., Pühler, A., and Kalinowski, J. (2002) Identification of two gene clusters in *Corynebacterium glutamicum* subspecies (39). *Involvement of bacterial peroxiredoxins. EMBO J.* 13, 453–461
12. Jordan, P. A., Tang, Y., Bradbury, A. J., Thomson, A. J., and Guest, J. R. (1998) Biochemical and spectroscopic characterization of *Escherichia coli* aconitases (AcnA and AcnB). *Biochem. J.* 344, 739–746
13. Emptage, M. H., Dreyers, J. L., Kennedy, M. C., and Beinert, H. (1983) Optical and EPR characterization of different species of active and inactive aconitase. *J. Biol. Chem.* 258, 11106–11111
14. Cunningham, L., Gruer, M. J., and Guest, J. R. (1997) Transcriptional regulation of the aconitase genes (acnA and acnB) of *Escherichia coli*. *Microbiology* 143, 3795–3805
15. Pang, T., Guest, J. R., Arntymuk, P. J., and Green, J. (2005) Switching aconitase between catalytic and regulatory modes involves iron-dependent dimer formation. *Mol. Microbiol.* 56, 1149–1158
16. Rouault, T. A., and Klausner, R. D. (1996) Iron-sulfur clusters as biosensors of oxidants and iron. *Trends Biochem. Sci.* 21, 174–177
17. Zheng, L., Kennedy, M. C., Beinert, H., and Zalkin, H. (1992) Mutual activation of active site residues in pig heart aconitase. *J. Biol. Chem.* 267, 7895–7893
18. Bryk, R., Griffin, P., and Nathan, C. (2000) Peroxynitrite reductase activity of bacterial peroxiredoxins. *Nature* 407, 211–215
19. Poole, R. K., and Hughes, M. N. (2000) New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. *Mol. Microbiol.* 36, 775–783
20. Yabuuchi, E., Kawamura, Y., Kosako, Y., and Ezaki, T. (1998) Emendation of genus *Achromobacter* and *Achromobacter xylosidans* (Yabuuchi and Yano) and proposal of *Achromobacter rublandii* (Packe and Vishniac) comb. nov., *Achromobacter piechaudii* (Kiredjian et al.) comb. nov., and *Achromobacter xylosidans* subsp. *denitrificans* (Rüger and Tan) comb. nov. *Microb. Immunol.* 42, 429–438
21. Mattoo, S., and Cherry, J. D. (2005) Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clin. Microbiol. Rev.* 18, 326–382