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Hydrogen Peroxide-Forming NADH Oxidase Belonging to the Peroxiredoxin Oxidoreductase Family: Existence and Physiological Role in Bacteria

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***Amphibacillus xylanus* and *Sporolactobacillus inulinus* NADH oxidases belonging to the peroxiredoxin oxidoreductase family show extremely high peroxide reductase activity for hydrogen peroxide and alkyl hydroperoxides in the presence of the small disulfide redox protein, AhpC (peroxiredoxin). In order to investigate the distribution of this enzyme system in bacteria, 15 bacterial strains were selected from typical aerobic, facultatively anaerobic, and anaerobic bacteria. AhpC-linked alkyl hydroperoxide reductase activities were detected in most of the tested strains, and especially high activities were shown in six bacterial species that grow well under aerobic conditions, including aerobic bacteria (*Alcaligenes faecalis* and *Bacillus licheniformis*) and facultatively anaerobic bacteria (*Amphibacillus xylanus*, *Sporolactobacillus inulinus*, *Escherichia coli*, and *Salmonella enterica* serovar Typhimurium). In the absence of AhpC, the purified enzymes from *A. xylanus* and *S. inulinus* catalyze the NADH-linked reduction of oxygen to hydrogen peroxide. Similar activities were observed in the cell extracts from each of these six strains. The cell extract of *B. licheniformis* revealed the highest AhpC-linked alkyl hydroperoxide reductase activity in the four strains, with V_{max} values for hydrogen peroxide and alkyl hydroperoxides being similar to those for the enzymes from *A. xylanus* and *S. inulinus*. Southern blot analysis of the three strains probed with the *A. xylanus* peroxiredoxin reductase gene revealed single strong bands, which are presumably derived from the individual peroxiredoxin reductase genes. Single bands were also revealed in other strains which show high AhpC-linked reductase activities, suggesting that the NADH oxidases belonging to the peroxiredoxin oxidoreductase family are widely distributed and possibly play an important role both in the peroxide-scavenging systems and in an effective regeneration system for NAD in aerobically growing bacteria.**

NADH oxidases are found in several microorganisms (9, 12, 20, 31, 41) and have been purified from at least nine bacterial species (7, 10, 13, 25, 30, 34, 40, 42, 44). There are two types of NADH oxidase, H_2O forming and hydrogen peroxide forming. We previously purified the hydrogen peroxide-forming NADH oxidases from aerobically grown *Amphibacillus xylanus* and *Sporolactobacillus inulinus*, both of which are facultatively anaerobic bacteria that lack a respiratory chain (25, 30). The physiological function of these enzymes was first thought to be the in vivo regeneration of NAD in aerobic metabolism of the bacteria (22–24, 30). The enzymes catalyze the reduction of oxygen by NADH to form hydrogen peroxide. However, in the presence of a 21-kDa disulfide-containing redox protein (AhpC), now commonly referred to as peroxiredoxin (Prx), the NADH oxidases also showed extremely high reductase activity for both hydrogen peroxide and alkyl hydroperoxides (26, 28–30). These NADH oxidases thus belong to a growing new family of peroxiredoxin oxidoreductases (PrxR) (38) and are involved not only in the regeneration of NAD but also in the removal of peroxides. Thus, in spite of lacking a respiratory chain and peroxide-scavenging enzymes such as catalase and heme-containing peroxidases, *Amphibacillus xylanus* and *Sporolactobacillus inulinus* can grow as well under aerobic conditions as do conventional aerobic bacteria (15, 24). In order to investigate the distribution of this type of NADH oxidase in bacteria, 15 bacterial strains were selected from aerobic, facultatively anaerobic, and anaerobic bacteria. The characteristics of the enzyme systems in these strains are reported here.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *Alcaligenes faecalis* NRIC 1001^T, *Pseudomonas aeruginosa* NRIC 1114^T, *Bacillus licheniformis* NRIC 1863, *Corynebacterium glutamicum* JCM 1318^T, *Escherichia coli* NRIC 1509, and *Salmonella enterica* serovar Typhimurium NRIC 1851 were grown under aerobic conditions with shaking. *Corynebacterium glutamicum* was cultured at 30°C, and *Alcaligenes faecalis*, *P. aeruginosa*, *Bacillus licheniformis*, *E. coli*, and *Salmonella enterica* serovar Typhimurium were cultured at 37°C. The growth medium of *Alcaligenes faecalis*, *Corynebacterium glutamicum*, and *Salmonella enterica* serovar Typhimurium consisted of 1.0% peptone, 1.0% beef extract, and 0.5% NaCl, pH 7.0. *P. aeruginosa* growth medium consisted of 0.63% peptone, 0.38% beef extract, and 0.63% NaCl, pH 7.0. *Bacillus licheniformis* growth medium consisted of 0.5% glucose, 0.2% yeast extract, 1.0% peptone, 0.2% ammonium nitrate, 0.1% sodium citrate, 0.6% KH_2PO_4, 1.4% K_2HPO_4, and 0.01% $MgSO_4 \cdot 7H_2O$, pH 7.0. *E. coli* growth medium consisted of 1.0% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.2. *Zymomonas mobilis* subsp. *mobilis* NRIC 1158^T was grown with or without shaking at 30°C in a medium consisting of 2.0% glucose and 0.5% yeast extract, pH 6.6. *Amphibacillus xylanus* JCM 7361^T was grown at 39.5°C with shaking for

TABLE 1. Bacterial strains used in this study[a]

Strain	Anaerobic growth	Aerobic growth	Respiratory chain[b]	Catalase[b] or peroxidase
Aerobic bacteria				
Alcaligenes faecalis NRIC 1001^T	−[b]	+	+	+
Pseudomonas aeruginosa NRIC 1114^T	−[b]	+	+	+
Bacillus licheniformis NRIC 1863	+[b]	+	+	+
Corynebacterium glutamicum JCM 1318^T	+[b]	+	+	+
Facultatively anaerobic bacteria having a respiratory chain				
Escherichia coli NRIC 1509	+[b]	+	+	+
Salmonella enterica serovar Typhimurium NRIC 1851	+[b]	+	+	+
Facultatively anaerobic bacteria lacking a respiratory chain				
Zymomonas mobilis subsp. *mobilis* NRIC 1158^T	+	+[c]	−	+
Amphibacillus xylanus JCM 7361^T	+[d]	+[d]	−	−
Sporolactobacillus inulinus NRIC 1133^T	+[e]	+[e]	−	−
Lactobacillus delbrueckii subsp. *delbrueckii* NRIC 1053^T	+	w[f]	−	+
Lactococcus lactis subsp. *lactis* NRIC 1149^T	+	+[c]	−	NT
Anaerobic bacteria				
Bacteroides vulgatus JCM 5826^T	+	−[b]	−	NT
Bifidobacterium bifidum JCM 1255^T	+	−[b]	−	NT
Clostridium aminovalericum DSM 1283^T	+	−[b]	−	NT
Clostridium butyricum JCM 1391^T	+	−[b]	−	NT

[a] NT, not tested, W, weak.
[b] Data from references 16 and 46.
[c] Data from this study.
[d] Data from reference 24.
[e] Data from reference 30.
[f] Data from reference 44.

aerobic growth or was grown anaerobically as described previously (24). (The type strain of *Amphibacillus xylanus* Ep01 was isolated as described previously [23] and has been deposited in the Japan Collection of Microorganisms, RIKEN, Wako, Saitama, Japan, as strain JCM 7361^T.) *Amphibacillus xylanus* growth medium consisted of 1.0% glucose, 0.3% yeast extract, 0.03% peptone, 0.2% ammonium nitrate, 0.1% K_2HPO_4, 1.0% $CaCl_2 \cdot 2H_2O$, and 1.0% salt solution containing 20 mg of $MgSO_4 \cdot 7H_2O$, 0.5 mg of $MnSO_4 \cdot 5H_2O$, and 0.5 mg of $FeSO_4 \cdot 7H_2O$ per ml. The medium was adjusted to pH 10.0 by 10% of 1 M $NaHCO_3$-Na_2CO_3 buffer, pH 10.55 (24). *Sporolactobacillus inulinus* NRIC 1133^T and *Lactobacillus delbrueckii* subsp. *delbrueckii* NRIC 1053^T were grown at 37 and 30°C, respectively, with shaking for aerobic growth or by flushing the medium bottles with nitrogen gas for anaerobic growth. *Lactococcus lactis* subsp. *lactis* NRIC 1149^T was grown at 30°C with or without shaking. The growth medium of *Sporolactobacillus inulinus*, *Lactobacillus delbrueckii*, and *Lactococcus lactis* consisted of 1.0% glucose, 1.0% yeast extract, 1.0% peptone, 1.0% sodium acetate, 0.2% beef extract, and 0.5% salt B solution containing 40 mg of $MgSO_4 \cdot 7H_2O$, 2 mg of $MnSO_4 \cdot 5H_2O$, 2 mg of $FeSO_4 \cdot 7H_2O$, and 2 mg of NaCl in 1 ml, pH 6.8. *Bacteroides vulgatus* JCM 5826^T, *Bifidobacterium bifidum* JCM 1255^T, *Clostridium aminovalericum* DSM 1283^T, and *Clostridium butyricum* JCM 1391^T were grown at 37°C by flushing the medium bottles with nitrogen gas for anaerobic growth; in addition, for aerobic growth, anaerobically growing cells were cultured with agitation for 15 min under aerobic conditions. The growth medium of *Bacteroides vulgatus*, *Clostridium aminovalericum*, and *Clostridium butyricum* consisted of 1.5% glucose, 1.0% yeast extract, 2.0% peptone, 0.22% beef extract, 0.5% soluble starch, 0.25% KH_2PO_4, 0.3% NaCl, 0.03% L-cysteine-HCl, 0.03% sodium thioglycolate, and 0.0001% resazurin, pH 7.3. The growth medium of *Bifidobacterium bifidum* consisted of 1.0% glucose, 0.5% yeast extract, 1.0% peptone, 0.5% beef extract, 0.1% Tween 80, 0.3% K_2HPO_4, 0.05% L-cysteine-HCl, and 1.0% sodium ascorbate, pH 6.8. All strains were harvested by centrifugation, washed with 50 mM sodium phosphate buffer, pH 7.0, containing 5 mM EDTA, and then stored at −80°C until use.

Preparation of cell extracts. Bacterial cells were suspended in three volumes of 50 mM sodium phosphate buffer, pH 7.0, containing 5 mM EDTA. The suspensions were treated with lysozyme and then disrupted by three passages through a French pressure cell (SLM-AMINCO; Spectronic Instruments) at 1,400 kg/cm^2. Phenylmethylsulfonyl fluoride (final concentration, 2 mM) was added immediately after the first passage through the French pressure cell. The supernatant was centrifuged at 16,000 × *g* for 20 min, the pellet discarded and the supernatant ultracentrifuged at 187,000 × *g* for 90 min to obtain the cytoplasmic fraction. The cytoplasmic fractions were dialyzed against 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA.

NADH oxidase assay. NADH oxidase activity was assayed at 30, 37, or 39.5°C (at the optimum growth temperature of each tested bacterium) in 3 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA, 0.25 mM NADH, and cell extracts. The reaction was monitored with a Clark oxygen electrode (model 5331; Yellow Springs Instrument Co., Yellow Springs, Ohio). One unit of activity was defined as the amount of protein required to catalyze the consumption of 1 μmol of oxygen per min.

Enzymes. NADH oxidases from *Amphibacillus xylanus* and from *Sporolactobacillus inulinus* were purified as described previously (30, 33). NADH oxidase from *Bacillus licheniformis* was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). AhpC from *A. xylanus* was isolated as described previously (29).

Southern blot analysis. The genomic DNA of all bacterial strains was isolated by the method of Saito and Miura (43). The DNA was digested with *Eco*RI, electrophoresed on an agarose gel, and then transferred to a positively charged nylon membrane. The membrane was hybridized with digoxigenin-11-dUTP-labeled random-primed *Amphibacillus* NADH oxidase DNA probes which were prepared using a DIG DNA labeling kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) according to the manufacturer's instructions.

Steady-state kinetics. The apparent K_m value for oxygen was determined from Lineweaver-Burk plots of the kinetic data obtained at 25°C and various oxygen concentrations in 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 120 μM NADH. The decrease in the A_{340} was monitored with a spectrophotometer.

Hydroperoxide reductase activity assays. The activities reported in Table 2 were obtained as follows. The reaction mixture (2 ml) containing 50 mM sodium phosphate buffer (pH 7.0), 2 mM EDTA, 4% ammonium sulfate, 2.5 mM NADH, 1 mM *t*-butyl hydroperoxide, cell extracts, and 30 μM *Amphibacillus xylanus* AhpC was loaded into a 2-ml microtube, which was closed with a cap. Alkyl hydroperoxide reduction was performed by incubation for 60 min at 30, 37, or 39.5°C (optimum growth temperatures of the individual bacteria). The reaction mixture was then subjected to high-performance liquid chromatographic analysis on an Inertsil ODS-2 column, with 5 mM potassium phosphate buffer (pH 7.0)-acetonitrile (9:1) as the mobile phase, and the absorbance of hydroperoxide at 230 nm was monitored. Activity was expressed in milliunits per milligram, where 1 mU is defined as reduction of 1 nmol of hydroperoxide per min.

Turnover studies of hydrogen peroxide or alkyl hydroperoxide reductase activities with the pure *Bacillus licheniformis* enzyme were carried out in a tem-

TABLE 2. *t*-Butyl hydroperoxide reductase activities in tested bacterial strains

Strain	*t*-Butyl hydroperoxide reductase activity (mU/mg)	
	No added AhpC	With added AhpC
Aerobic bacteria		
Alcaligenes faecalis NRIC 1001^T	0.3	14.3
Pseudomonas aeruginosa NRIC 1114^T	0.3	1.4
Bacillus licheniformis NRIC 1863	4.7	51.1
Corynebacterium glutamicum JCM 1318^T	2.3	2.4
Facultatively anaerobic bacteria with a respiratory chain		
Escherichia coli NRIC 1509	1.4	8.8
Salmonella enterica serovar Typhimurium NRIC 1851	2.8	6.8
Facultatively anaerobic bacteria lacking a respiratory chain		
Zymomonas mobilis NRIC 1158^T	1.3	2.1
Amphibacillus xylanus JCM 7361^T	8.3	583.0
Sporolactobacillus inulinus NRIC 1133^T	0.2	8.6
Lactobacillus delbrueckii NRIC 1053^T	0.7	1.8
Lactococcus lactis NRIC 1149^T	4.4	5.4
Anaerobic bacteria		
Bacteroides vulgatus JCM 5826^T	2.4	2.43
Bifidobacterium bifidum JCM 1255^T	0.8	2.8
Clostridium aminovalericum DSM 1283^T	3.8	5.5
Clostridium butyricum JCM 1391^T	2.1	3.7

perature-controlled stopped-flow spectrophotometer (Hi-tech SF-61) interfaced with a Dell 325D computer. The activity was measured under anaerobic conditions as described previously (26).

RESULTS

Distribution of alkyl hydroperoxide reductase activity in the presence of AhpC in bacteria. The AhpC-linked alkyl hydroperoxide reductase activities in cell extracts of bacteria were examined using *t*-butyl hydroperoxide as the substrate. By high-performance liquid chromatographic analysis, the reduction of *t*-butyl hydroperoxide was assayed in the presence of various combinations of the cell extract, AhpC from *A. xylanus*, and NADH. Although in the absence of additional AhpC, every bacterial cell extract showed some reductase activity for alkyl hydroperoxide, the activity was increased by the addition of *A. xylanus* AhpC in most of the tested strains. Since AhpC proteins are widely distributed in bacteria, including aerobic, facultatively anaerobic, and anaerobic bacteria, the cell extracts of each tested strain presumably contain some AhpC protein (2, 3, 5, 19, 21). In the preparation and assay of the cell extracts, the concentrations of endogenous AhpC were diluted about 15-fold and may not have been high enough for the full observation of AhpC-linked alkyl hydroperoxide reductase activity compared with that in vivo, suggesting that the observed increments of activity by addition of AhpC can be attributed to intrinsic AhpC-linked hydroperoxide reductase activity. AhpC-linked *t*-butyl hydroperoxide reductase activities were detected in most of the tested strains (Table 2). Especially high increments of activity (over 4.0 mU/mg more than that found for the archetypal *Salmonella enterica* serovar Typhimurium) were observed in six species, including the aerobic bacteria: *Alcaligenes faecalis* and *Bacillus licheniformis*, facultatively anaerobic bacteria having a respiratory chain (*E. coli* and *Salmonella enterica* serovar Typhimurium), and facultatively anaerobic bacteria lacking a respiratory chain (*Amphibacillus xylanus* and *Sporolactobacillus inulinus*). The two aerobic bacteria and two of the facultatively anaerobic bacteria have a respiratory chain and also catalase. The other two species belonging to the facultatively anaerobic class lack a respiratory chain and also lack catalase. These data suggest that the AhpC-linked reductase system functions as an effective system for removing hydroperoxides in bacteria growing under aerobic conditions, whether the bacteria have or lack catalase (see Discussion).

Distribution of hydrogen peroxide-forming NADH oxidase activity in bacteria. NADH oxidase activity is defined as the ability to reduce oxygen with NADH, regardless of whether the reduction product is H_2O_2 or H_2O. Cell extracts of bacteria with a respiratory chain also show oxygen consumption caused by the addition of NADH, but this consumption should be inhibited by KCN, while the oxygen consumption by NADH oxidases is not (25, 30). Oxygen consumption on the addition of NADH was observed in cell extracts of all strains used in this study (Table 3). These activities were in general not inhibited or, in some cases, only marginally inhibited by KCN (1 mM), suggesting that NADH oxidases were present in all of the tested strains (Table 3). NADH oxidases so far studied are divided into two types, hydrogen peroxide- and H_2O-forming enzymes, both of which have been found in several bacterial species (1, 7, 10, 13, 25, 30, 34, 40–42, 44). Hydrogen peroxide-forming NADH oxidases are known to be markedly accelerated in activity by addition of free flavin adenine dinucleotide (FAD) (10, 30, 33). In contrast, NADH oxidase activity by addition of free FAD is only increased slightly with a H_2O-forming NADH oxidase (10). NADH oxidase activities which were accelerated over fivefold by the addition of 50 μM FAD were observed in strains of the following species: *Alcaligenes faecalis* and *Bacillus licheniformis* (aerobic bacteria); *E. coli* and *Salmonella enterica* serovar Typhimurium (facultatively anaerobic bacteria having a respiratory chain); *Amphibacillus xylanus*, *Sporolactobacillus inulinus*, and *Lactobacillus delbrueckii* (facultatively anaerobic bacteria lacking a respiratory chain); and the anaerobic bacterium *Bacteroides vulgatus*. Hydrogen peroxide-forming NADH oxidase activity is thus presumably present in these eight strains, including the six strains showing high AhpC-linked *t*-butyl hydroperoxide reductase activities.

Reaction products in the absence or presence of AhpC. To confirm the presence of hydrogen peroxide-forming NADH oxidase in these eight strains, oxygen consumption experiments were performed with and without the addition of catalase. The purified alkyl hydroperoxide reductase flavoprotein of *Amphibacillus xylanus*, which catalyzed the reduction of oxygen to hydrogen peroxide, showed 50% reformation of oxygen on addition of catalase (25); however, in the presence of AhpC no reformation of oxygen was detected on addition of catalase (29). The enzyme thus catalyzes the four-electron reduction of oxygen to water in the presence of additional AhpC. Even in cell extract experiments with *A. xylanus*, in which the presence of AhpC has been shown unambiguously (29), hydrogen peroxide is found to accumulate on oxidation of NADH, as shown

TABLE 3. NADH oxidase activities in tested bacterial species

Strains	NADH oxidase activity (mU/mg)			Ratio of activity +FAD/−FAD (no added KCN)
	−FAD		+FAD, −KCN	
	−KCN	+KCN		
Aerobic bacteria				
Alcaligenes faecalis NRIC 1001^T	1.4	1.7	13.3	9.5
Pseudomonas aeruginosa NRIC 1114^T	3.0	3.5	11.5	3.8
Bacillus licheniformis NRIC 1863	10.0	6.1	266	26.6
Corynebacterium glutamicum JCM 1318^T	1.1	1.0	2.9	2.6
Facultatively anaerobic bacteria with a respiratory chain				
Escherichia coli NRIC 1509	2.2	2.6	12.7	5.8
Salmonella enterica serovar Typhimurium NRIC 1851	1.9	2.3	16.5	8.7
Facultatively anaerobic bacteria lacking a respiratory chain				
Zymomonas mobilis NRIC 1158^T	1.9	2.1	4.0	2.1
Amphibacillus xylanus JCM 7361^T	54.7	56.7	347	6.3
Sporolactobacillus inulinus NRIC 1133^T	9.7	4.5	79.2	8.2
Lactobacillus delbrueckii NRIC 1053^T	7.6	6.5	181	23.8
Lactococcus lactis NRIC 1149^T	20.8	5.6	39.5	1.9
Anaerobic bacteria				
Bacteroides vulgatus JCM 5826^T	2.4	1.9	13.9	5.8
Bifidobacterium bifidum JCM 1255^T	7.7	7.5	23.2	3.0
Clostridium aminovalericum DSM 1283^T	8.0	6.8	18.4	2.3
Clostridium butyricum JCM 1391^T	11.1	8.5	25.4	2.3

by O_2 liberation on addition of catalase, but does not accumulate in the presence of added AhpC (data not shown). In the cell extracts from *Sporolactobacillus inulinus*, *Lactobacillus delbrueckii*, and *Bacteroides vulgatus*, reformation of oxygen on addition of catalase was observed in the absence of AhpC, and in the presence of AhpC, no hydrogen peroxide production was observed (results not shown). These results show that a hydrogen peroxide-forming NADH oxidase was present and could function as a peroxide reductase in these bacteria in the presence of AhpC. All of these bacteria lack catalase. As expected, in the absence of AhpC, reformation of oxygen was not observed in the cell extracts from bacterial species having catalase: *Alcaligenes faecalis*, *Bacillus licheniformis*, *E. coli*, and *Salmonella enterica* serovar Typhimurium. Since acceleration of NADH oxidase activity on addition of FAD indicates the presence of hydrogen peroxide-forming NADH oxidases in these bacteria, hydrogen peroxide produced by the NADH oxidase reaction is presumably scavenged by the endogenous catalase.

Southern blot analysis. To demonstrate the distribution of the *prxR* gene in bacteria, we performed Southern blot analysis of genomic DNA of the tested bacterial strains which had been digested with *Eco*RI and probed with *Amphibacillus xylanus* *prxR*. Southern blot analysis revealed a single band in six bacterial strains (Fig. 1). Weak bands were revealed in *Alcaligenes faecalis*, *E. coli*, *Clostridium aminovalericum*, and *Bacteroides vulgatus*. Particularly strong bands were revealed in *Bacillus licheniformis* and *Sporolactobacillus inulinus*, indicating that the DNA fragment, which has a high degree of similarity to the *Amphibacillus xylanus* NADH oxidase gene, is in the nuclear DNA of *Bacillus licheniformis* and *Sporolactobacillus inulinus*. In these bacteria, including *Amphibacillus xylanus*, as mentioned above, marked acceleration of NADH oxidase activity on addition of FAD is observed, and high AhpC-linked *t*-butyl hydroperoxide reductase activity is also observed in the presence of AhpC, suggesting that the NADH oxidases from these bacteria have enzymatic properties similar to those of enzymes from *Amphibacillus xylanus*, i.e., they are all members of the peroxiredoxin oxidoreductase (PrxR) family. A comparison of the purified NADH oxidases from these bacteria is presented below.

Characterization of purified hydrogen peroxide forming-NADH oxidases from *Amphibacillus xylanus*, *Bacillus lichenifor-*

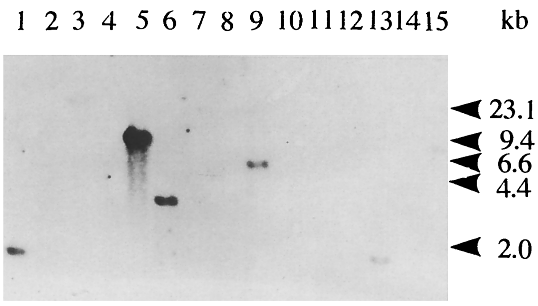

FIG. 1. Southern blot analysis of the DNA of tested bacterial strains. The DNAs were digested with *Eco*RI. Hybridization was done as described in Materials and Methods. Lane 1, *Bacillus licheniformis* NRIC 1863; lane 2, *Corynebacterium glutamicum* JCM 1318^T; lane 3, *Alcaligenes faecalis* NRIC 1001^T; lane 4, *P. aeruginosa* NRIC 1114^T; lane 5, *Amphibacillus xylanus* JCM 7361^T; lane 6, *Sporolactobacillus inulinus* NRIC 1133^T; lane 7, *Lactobacillus delbrueckii* subsp. *delbrueckii* NRIC 1053^T; lane 8, *Lactococcus lactis* subsp. *lactis* NRIC 1149^T; lane 9, *E. coli* NRIC 1509; lane 10, *Salmonella enterica* serovar Typhimurium NRIC 1851; lane 11, *Z. mobilis* subsp. *mobilis* NRIC 1158^T; lane 12, *Bifidobacterium bifidum* JCM 1255^T; lane 13, *Clostridium aminovalericum* DSM 1283^T; lane 14, *Clostridium butyricum* JCM 1391^T; lane 15, *Bacteroides vulgatus* JCM 5826^T. DNA size markers are on the right.

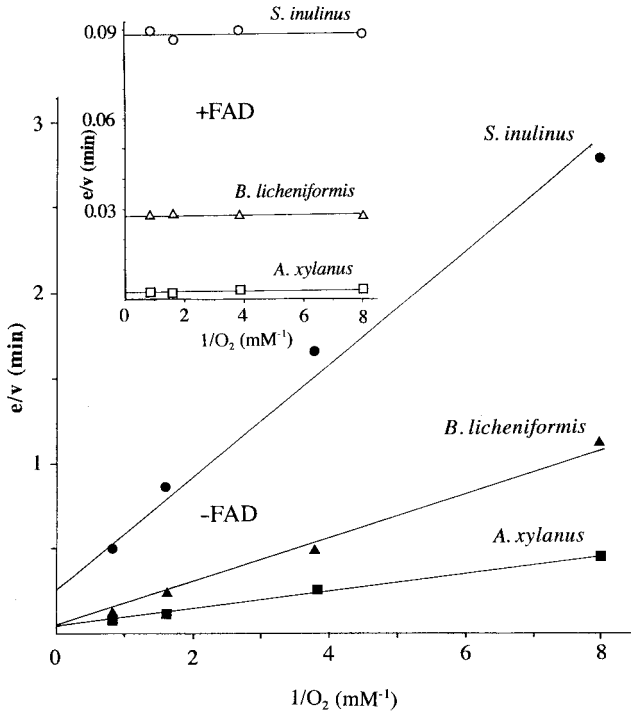

FIG. 2. Lineweaver-Burk plots of steady-state kinetic analyses of NADH oxidases from the indicated species. Assay conditions were 50 mM sodium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 120 μM NADH with or without 150 μM FAD at 25°C.

***mis*, and *Sporolactobacillus inulinus*.** In our previous reports of *Amphibacillus xylanus* NADH oxidase, the K_m value for oxygen was found to be 1.7 mM, too high to catalyze effectively the reoxidation of NADH by oxygen in the cell (33). In the presence of additional FAD, the NADH oxidase activity was accelerated markedly, and the K_m value for oxygen was greatly diminished; indeed, it was too low to allow accurate determination of its value by the usual assay method (33). The NADH oxidase activity in cell extracts from *Bacillus licheniformis* and *Sporolactobacillus inulinus* was also found to be accelerated in the presence of additional FAD. Further analyses were carried out with the purified enzymes. Lineweaver-Burk plots of the NADH oxidase activities in the presence of 120 μM NADH are shown in Fig. 2. The apparent K_m values for oxygen with the NADH oxidases from *Bacillus licheniformis* and from *Sporolactobacillus inulinus* were 3.3 and 1.3 mM, respectively. The Lineweaver-Burk plots of the NADH oxidase activities from these strains were similar to that of *Amphibacillus xylanus* PrxR. In the presence of FAD, the NADH oxidase activities were accelerated, and the apparent K_m values for oxygen were greatly diminished and were too low to allow accurate determination of values (Fig. 2, inset).

Amphibacillus xylanus NADH oxidase and *Sporolactobacillus inulinus* NADH oxidase showed extremely high reductase activity for both hydrogen peroxide and alkyl hydroperoxide in the presence of AhpC (28–30). Reductase activity assays of *Bacillus licheniformis*, using hydrogen peroxide and cumene hydroperoxide as substrates under anaerobic conditions, were carried out in the presence or absence of AhpC. In the absence of AhpC, no peroxide reductase activity was observed. In contrast, hydrogen peroxide and cumene hydroperoxide were rapidly reduced in the presence of AhpC. The extrapolated V_{max} values for hydrogen peroxide and cumene hydroperoxide reductase activities of the *Bacillus* NADH oxidase were 9,200 and 9,300 min^{-1}, respectively. These V_{max} values are similar to those of the peroxiredoxin reductases from *Amphibacillus xylanus* and *Sporolactobacillus inulinus* and slightly lower than that from *Salmonella enterica* serovar Typhimurium (Table 4).

TABLE 4. Peroxide reductase activities of purified NADH oxidase and alkyl hydroperoxide reductase in the presence of AhpC

Enzyme	V_{max} (min^{-1})	
	Hydrogen peroxide	Cumene hydroperoxide
Amphibacillus xylanus NADH oxidase[a]	8,900	8,900
Bacillus licheniformis NADH oxidase	9,200	9,300
Sporolactobacillus inulinus NADH oxidase[b]	9,300	8,300
Alkaliphilic *Bacillus* NADH dehydrogenase[c]	8,300	10,000
Salmonella enterica serovar Typhimurium alkyl hydroperoxide reductase[d]	14,900	14,300

[a] Data from reference 29.
[b] Data from reference 30.
[c] Data from reference 14.
[d] Data from reference 26.

Induction of NADH oxidase activity under aerobic conditions. Nine bacterial species, including facultatively anaerobic bacteria lacking a respiratory chain (*Z. mobilis*, *Amphibacillus xylanus*, *Sporolactobacillus inulinus*, *Lactobacillus delbrueckii*, and *Lactococcus lactis*) and anaerobic bacteria (*Bacteroides vulgatus*, *Bifidobacterium bifidum*, *Clostridium aminovalericum*, and *Clostridium butyricum*), were cultivated under anaerobic or aerobic conditions in this study. The NADH oxidase activities which were induced over twofold under aerobic conditions compared to under anaerobic conditions were observed only in the facultatively anaerobic bacteria lacking a respiratory chain: *Z. mobilis*, *Amphibacillus xylanus*, *Sporolactobacillus inulinus*, and *Lactobacillus lactis* (Table 5). Induction of NADH oxidase activities over fourfold were especially evident in *Amphibacillus xylanus* and *Sporolactobacillus inulinus*, both of which can

TABLE 5. NADH oxidase activities in tested strains under anaerobic or aerobic conditions

Strain	NADH oxidase activity (mU/mg)	
	Anaerobic growth	Aerobic growth
Facultatively anaerobic bacteria lacking a respiratory chain		
Zymomonas mobilis NRIC 1158^T	0.8	1.9
Amphibacillus xylanus JCM 7361^T	8.9	54.7
Sporolactobacillus inulinus NRIC 1133^T	2.1	9.7
Lactobacillus delbrueckii NRIC 1053^T	7.0	7.6
Lactococcus lactis NRIC 1149^T	8.5	20.8
Anaerobic bacteria		
Bacteroides vulgatus JCM 5826^T	2.4	3.3
Bifidobacterium bifidum JCM 1255^T	7.7	8.0
Clostridium aminovalericum DSM 1283^T	8.0	9.6
Clostridium butyricum JCM 1391^T	11.1	11.8

grow well under aerobic conditions as well as under anaerobic conditions. However, induction of the NADH oxidase activities was not observed in anaerobic bacteria, which cannot grow well under aerobic conditions, including *Bacteroides vulgatus*, *Bifidobacterium bifidum*, *Clostridium aminovalericum*, and *Clostridium butyricum* (Table 5). The induced NADH oxidase is thus possibly playing an important role in aerobic metabolism of the bacteria (see below).

DISCUSSION

AhpC-linked *t*-butyl hydroperoxide reductase activities were observed in cell extracts from most of the tested strains (Table 2). The bacteria growing well under aerobic conditions, except *Corynebacterium glutamicum* and *P. aeruginosa*, showed high AhpC-linked reductase activity. In contrast, AhpC-linked *t*-butyl hydroperoxide reductase activities were low in bacteria which cannot grow well under aerobic conditions. These results suggest that the peroxiredoxin oxidoreductase system is present mainly in bacteria capable of good aerobic growth. Although *Corynebacterium glutamicum* and *P. aeruginosa* grow well under aerobic conditions, high AhpC-linked *t*-butyl hydroperoxide reductase activities were not observed in their cell extracts. The NADH oxidase activities of enzymes belonging to the peroxiredoxin reductase family are markedly accelerated on addition of free FAD (30, 33), but the NADH oxidase activities of the above two organisms were only slightly accelerated by addition of FAD, suggesting that the content of peroxiredoxin oxidoreductase is low or absent in these strains. Conventional heme peroxidases or catalase probably function to remove peroxides in such bacteria.

All of the tested strains showing PrxR activity in the cell extracts, except for *Salmonella enterica* serovar Typhimurium, revealed a single band in Southern blot analyses probed with the *Amphibacillus xylanus prxR* gene. *Salmonella enterica* serovar Typhimurium was the first organism shown to possess the peroxiredoxin reductase-peroxiredoxin system (11) and the nucleotide sequence of the gene encoding the flavoprotein component, *prxR* (formerly known as AhpF), has already been determined (48). Regardless of their similar catalytic function, the similarity of their nucleotide sequences is presumably not sufficient (54.8%) for rigorous hybridization. Particularly strong bands were revealed in the tested gram-positive bacteria, including *Amphibacillus xylanus*, *Sporolactobacillus inulinus*, and *Bacillus licheniformis*, suggesting high similarity in their nucleotide sequences. In a previous article, we reported that *Sporolactobacillus* NADH oxidase catalyzes the reduction of oxygen to hydrogen peroxide and, in the presence of AhpC, of hydrogen peroxide to water (30). The purified enzyme from *Bacillus licheniformis* also showed high reductase activity for both hydrogen peroxide and alkyl hydroperoxide. Their V_{max} values were similar to those of the NADH oxidases from *Amphibacillus xylanus* and *Sporolactobacillus inulinus* (Table 4).

The AhpC-linked two-electron reduction of hydrogen peroxide and alkyl hydroperoxides has been observed with the following enzymes: NADH oxidases from *Amphibacillus xylanus* (26, 28, 29), *Sporolactobacillus inulinus* (30), *Streptococcus mutans* (39), and *Bacillus licheniformis* (this work); NADH dehydrogenase from an alkaliphilic *Bacillus* strain (14); and alkyl hydroperoxide reductase flavin component (AhpF) from *Salmonella enterica* serovar Typhimurium (26, 36, 37, 38). All of these enzyme systems belong to the peroxiredoxin oxidoreductase family and show extremely high reductase activities for both hydrogen peroxide and alkyl hydroperoxides (14, 26, 28–30). Their V_{max} values are similar to the rate constant for reduction of the flavin component of the *Amphibacillus* PrxR by NADH, suggesting that this was the rate-determining step of the overall reaction (27). The K_m values for hydroperoxides were too low to allow accurate determination of their values in our experiments. No other peroxide-scavenging enzyme, catalase or peroxidase, so far studied has been reported to show such high turnover numbers and low K_m values for both hydrogen peroxide and alkyl hydroperoxide as described here (4, 6, 8, 11, 17, 32, 35, 47). A recent report also describes the isolation and function of peroxiredoxin and peroxiredoxin reductase from a thermophile, *Thermus aquaticus* (18). The flavoprotein component, PrxR, was originally isolated as an NADH oxidase (7). This system, however, shows much lower catalytic activity than the ones described above (18).

Amphibacillus xylanus and *Sporolactobacillus inulinus*, lacking both catalase and conventional heme-containing peroxidase, can grow as well under aerobic conditions as aerobic bacteria that have catalase and peroxidase, suggesting that the PrxR-Prx system can function in the removal of peroxides in these organisms. The NADH oxidases from these organisms thus catalyze the four-electron reduction of oxygen to water in the presence of AhpC (28, 30). This activity is also thought to function in vivo to regenerate NAD from NADH produced in aerobic metabolism of *Amphibacillus xylanus* and *Sporolactobacillus inulinus*, both of which lack a respiratory chain (Fig. 3) (28–30). On the other hand, the NADH oxidase activities of *Salmonella* AhpF and *Bacillus* NADH dehydrogenase are low compared with those of enzyme from *Amphibacillus xylanus* and *Sporolactobacillus inulinus*. The PrxR-Prx systems presumably function physiologically to remove peroxides in *Salmonella* and *Bacillus*, because these organisms have a respiratory chain and do not need the oxidase activity for the regeneration of NAD. Indeed, the only functional difference in the NADH-linked flavoprotein components appears to be in the low reactivities of the *Salmonella* and *Bacillus* enzymes with molecular oxygen. Acceleration of NADH oxidase activities by addition of FAD and AhpC-linked peroxide reductase activities was widely detected in most of the aerobically growing bacteria, indicating that the AhpC-linked NADH oxidase system is distributed widely not only in facultative anaerobes that lack a respiratory chain but also in aerobes having a respiratory chain. This conclusion has also been reached on the basis of BLAST searches of GenBank and other databases, indicating a wide distribution of PrxR and Prx proteins (38).

In conclusion, the NADH oxidases belonging to the peroxiredoxin oxidoreductase family are widely distributed and probably play an important role both in peroxide-scavenging systems and in effective regeneration of NAD to maintain oxidative and reductive balance in bacterial cells that can grow well under aerobic conditions, including not only bacteria lacking a respiratory chain, catalase, and conventional peroxidases but also bacteria having all of these.

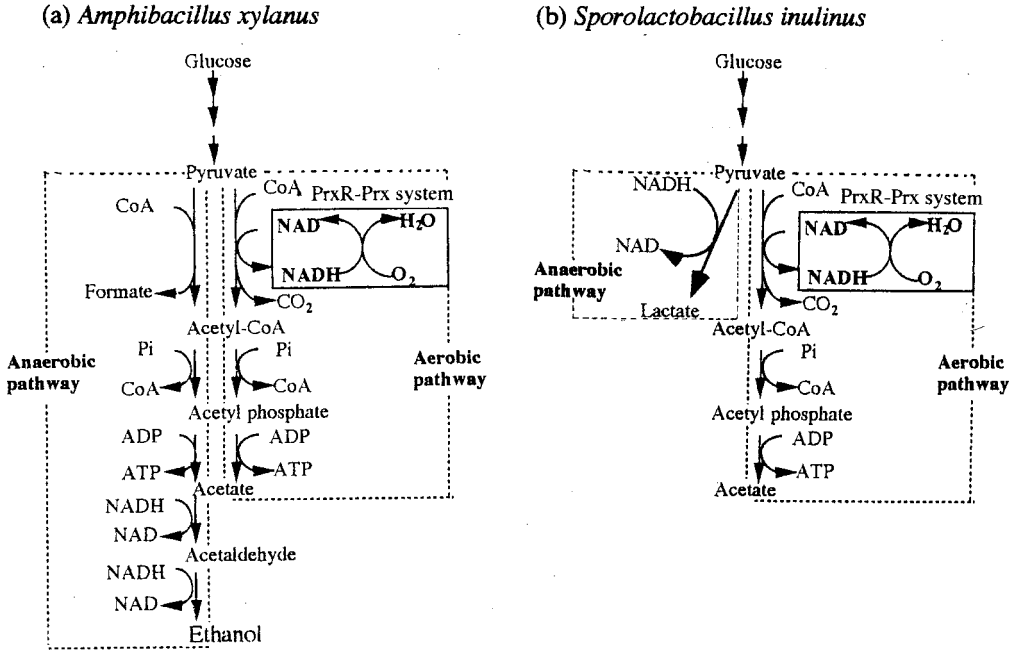

FIG. 3. Metabolic pathways of *Amphibacillus xylanus* and *Sporolactobacillus inulinus*.

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