The Journal of Microbiology (2010) Vol. 48, No. 6, pp. 778-783
DOI 10.1007/s12275-010-0149-z

Role of Hydrogen Generation by *Klebsiella pneumoniae* in the Oral Cavity

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(Received April 16, 2010 / Accepted August 11, 2010)

Some gastrointestinal bacteria synthesize hydrogen (H_2) by fermentation. Despite the presence of bactericidal factors in human saliva, a large number of bacteria also live in the oral cavity. It has never been shown that oral bacteria also produce H_2 or what role H_2 might play in the oral cavity. It was found that a significant amount of H_2 is synthesized in the oral cavity of healthy human subjects, and that its generation is enhanced by the presence of glucose but inhibited by either teeth brushing or sterilization with povidone iodine. These observations suggest the presence of H_2-generating bacteria in the oral cavity. The screening of commensal bacteria in the oral cavity revealed that a variety of anaerobic bacteria generate H_2. Among them, *Klebsiella pneumoniae* (*K. pneumoniae*) generated significantly large amounts of H_2 in the presence of glucose. Biochemical analysis revealed that various proteins in *K. pneumoniae* are carbonylated under standard culture conditions, and that oxidative stress induced by the presence of Fe^{++} and H_2O_2 increases the number of carbonylated proteins, particularly when their hydrogenase activity is inhibited by KCN. Inhibition of H_2 generation markedly suppresses the growth of *K. pneumoniae*. These observations suggest that H_2 generation and/or the reduction of oxidative stress is important for the survival and growth of *K. pneumoniae* in the oral cavity.

***Keywords*:** hydrogen, hydrogenase, oral cavity, *K. pneumoniae*

Since the oral cavity is exposed to a large number of pathogens, neutrophils and macrophages constitutively infiltrate the oral mucosal surface of healthy human subjects (Yamamoto *et al.*, 1991; Nakahara *et al.*, 1998; Sato *et al.*, 2003, 2008). Neutrophils in the oral cavity are activated under physiological conditions to release reactive oxygen species (ROS) and nitric oxide (NO) (Nakahara *et al.*, 1998; Sato *et al.*, 2003; Choudhury *et al.*, 2007; Sato *et al.*, 2008). Because ROS and NO are constitutively generated in the oral cavity, oral bacteria have developed defense systems against these toxic metabolites. Antioxidant enzymes, such as superoxide dismutase and catalase, are components of these survival systems. A variety of bacteria living in the oral cavity and gastrointestinal tract, such as *Helicobacter pylori*, *Streptococcus mutans*, and *Enterococcus faecalis*, reduce the toxic effects of NO by generating superoxide radicals (Nagata *et al.*, 1998; Nakamura *et al.*, 2000; Sato *et al.*, 2008; Nishikawa *et al.*, 2009). Since superoxide, NO and peroxynitrite (a reaction product of these two radicals) play important roles in the oxidative damage of a variety of cells, these bacteria should have potent mechanisms for the reduction of oxidative stress caused by reactive species.

Hydrogen reduces oxidative damage to cells and the brain injury caused by occlusion of the middle cerebral artery in rats (Ohsawa *et al.*, 2007). Inhalation of hydrogen gas also suppresses post-ischemic reperfusion injury of the mouse liver (Fukuda *et al.*, 2007). Oral administration of hydrogen-enriched water reduces oxidative injury of the mouse brain caused by physical restraint stress and prevents a decline in learning memory (Nagata *et al.*, 2009). Intake of hydrogen-enriched water also appears to improve lipid and glucose metabolism in patients with type 2 diabetes (Kajiyama *et al.,* 2008). Although these observations are suggestive of the beneficial effects of hydrogen against disease, its effect on the metabolism and survival of oral bacteria has not been determined. Hydrogen is generated by several mechanisms, including a hydrogenase-catalized reaction, and is consumed by some anaerobic bacteria to synthesize ATP (Adams, 1990; Adams and Stiefel, 1998; De Lacey *et al.*, 2007). Therefore, H_2 generation might be critically important for the survival of certain bacteria in oxidative environments. This study examined the role of H_2 generation in the survival of bacteria, such as *Klebsiella pneumoniae*, in the human oral cavity.

Materials and Methods

Bacterial cultures

Klebsiella pneumoniae (ATCC52145 and H79192), *Staphylococcus aureus* (DSM346), *Heamophilus influenzae* (ATCC9334), and *Neisseria meningitidis* (IP6764) were grown overnight on BHI-based chocolate plates at 37°C and transferred to BHI broth (pH 7.7; Difco, USA) for another overnight incubation at 37°C. Cells (OD=0.1 at 660 nm) were inoculated into 5 or 50 ml of BHI (1×10^7 - 2×10^9 cells/ml, OD_{660}=0.7-2.4) and cultured with shaking at 37°C for 3-5 h before they were used

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in experiments. *Escherichia coli* (DH5α, LB20) was grown in LB overnight at 37°C. Cells (OD_{660}=0.1) were inoculated into 5 ml LB broth, cultured with shaking at 37°C for 3-5 h, and then used in experiments.

Analysis of H_2 generation

Hydrogen generation by bacteria was measured using a Biogas H_2 Analyzer BAS-200 (Mitoleben, Japan). Volunteers placed the H_2 sensor on their opened mouths and breathed out toward the sensor (5-sec measurement). H_2 generated by *K. pneumoniae* (1×10^7 cells/ml, log phase) cultured in a test tube was measured by placing the H_2 sensor on the mouth of the tube (5-sec measurement).

Extraction of DNA from human saliva

Saliva (1 ml) was collected from six healthy volunteers after obtaining informed consent. DNA of salivary specimens was purified using the UltraCleanTM Soil DNA Isolation kit (MO BIO, USA) according to the manufacturer's instructions.

Analysis of 16S rRNA and hydrogenase in *K. pneumoniae*

DNA extracted from *K. pneumoniae* was PCR-amplified using primers for 16S rRNA [forward: 5′-AGCACAGAGAGCTTG-3′; reverse: 5′-ACTTTGGTCTTGCGAC-3′] and hydrogenase [forward: 5′-GGTCA ACACTACCTTGCG-3′; reverse: 5′-ACTGAACACCACCGTCTC-3′]. Each 50 μl PCR reaction contained 5 μl of 10× Taq buffer, 4 μl of 2.5 mM dNTP mixture (deoxynucleoside triphosphate), 1 μl of the primer and 5 μl of DNA template using 0.25 μl of Taq DNA polymerase (Takara Co, Japan). Aliquots of each PCR product (4 μl) were subjected to 2% agarose gel electrophoresis.

Analysis of protein carbonyls

Oxidatively modified proteins in *K. pneumoniae* were analyzed by immunoblotting as previously described (Park *et al.*, 2004). Briefly, cells were disrupted in 10 mM sodium phosphate buffer (pH 7.4) containing 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM deferoxamine in the presence of 2% SDS at 4°C and centrifuged at 15,000×g for 10 min. 2,4-dinitrophenylhydrazine (8 mM) was added to the supernatant fractions at 15°C for 60 min to react with the carbonyl groups in cellular proteins. The proteins conjugated with dinitrophenol (DNP) were electrophoresed in SDS-polyacrylamide gels followed by Western blot analysis using rabbit anti-DNP antibody (CHEMICON®, Japan) and an ECL Plus kit (GE Healthscience Co., Japan). Protein bands were stained with Coomassie Brilliant Blue R-250.

Results

Hydrogen generation

Under non-expirating conditions, the concentration of H_2 gas in the oral cavity of healthy volunteers was usually around 20-30 ppm (Fig. 1). When 1 ml of 10 mM glucose was administered into the oral cavity, H_2 levels rapidly increased up to 120 ppm, then slowly decreased and returned to initial levels. The rate of generation decreased markedly after teeth brushing and mouth washing. Under these conditions, the glucose-enhanced generation of H_2 was not observed. When the oral cavity was sterilized with 1% poly[(2-oxopyrrolidin-1-yl) ethylene] iodine, H_2 generation was strongly inhibited. These observations suggested the presence of H_2-generating bacteria in the oral cavity of healthy human subjects.

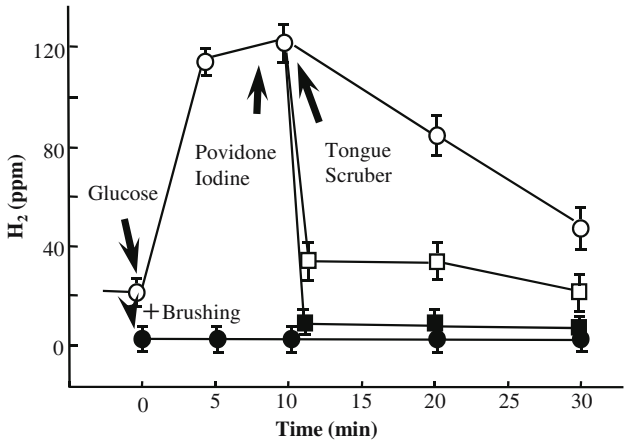


Fig. 1. H_2 generation in the oral cavity. Hydrogen levels in healthy human volunteers were measured by a Biogas H_2 Analyzer BAS-200 before and after adding 1 ml of 10 mM Glucose (○). Hydrogen generation was also measured after brushing teeth and washing the mouth (●), treating the oral cavity with 1% povidone iodine (■), and removing bacteria on the tongue with a scrubber (□). Data are shown as the Means±SD derived from three healthy volunteers.

Oral bacteria

Oral bacteria were examined for their ability to synthesize H_2. *K. pneumoniae*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* generated H_2 at 240, 48, and 30 ppm per sec, respectively. Other bacteria, including *Streptococcus mutans*, *E. coli*, *H. influenzae* and *N. meningitidis,* did not generate detectable amounts of H_2 (Fig. 2).

Analysis of *K. pneumoniae* hydrogenase in the oral cavity

To confirm that *K. pneumoniae* in the oral cavity generates H_2, DNA was extracted from salivary samples (1 ml) collected

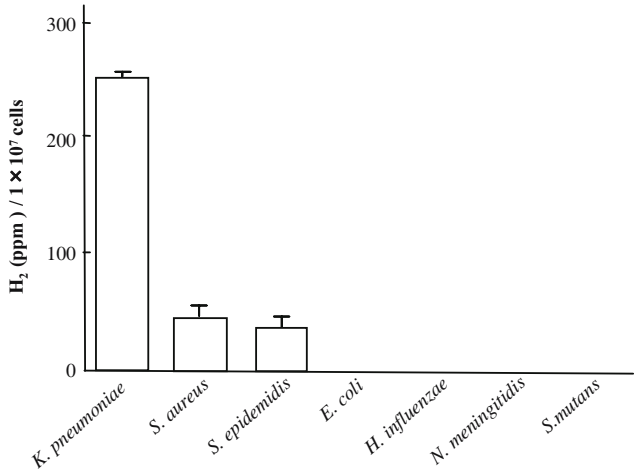
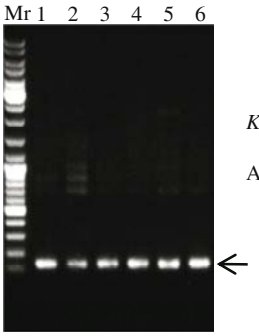


Fig. 2. Hydrogen generation by oral bacteria. *K. pneumoniae* and other bacteria were cultured on BHI-based chocolate plates at 37°C overnight and then incubated in BHI medium (pH 7.7) at 37°C overnight. The incubated cells (OD=0.1 at 660 nm) were inoculated into 5 ml BHI medium and cultured with shaking at 37°C for 3 h (1×10^7 cells/ml, log phase). Data are shown as the Means±SD derived from three separate experiments.



K. pneumoniae 16S rRNA primer
Sense: AGCACAGAGAGCTTG
Antisense: ACTTTGGTCTTGCGAC

Fig. 3. Detection of *K. pneumoniae* 16S rRNA. DNA was extracted and purified from 1 ml saliva of six healthy volunteers. PCR amplification was performed to detect Klebsiella 16S rRNA. Each PCR reaction was carried out in a 50 μl reaction buffer containing 0.25 μl of Taq DNA polymerase (TaKaRa), 5 μl of 10× Taq buffer, 4 μl of 2.5 mM dNTP mixture (deoxynucleoside triphosphate), 1 μl of the selected primer and 5 μl of DNA template. Aliquots (4 μl) of each PCR products were subjected to 2% agarose gel electrophoresis.

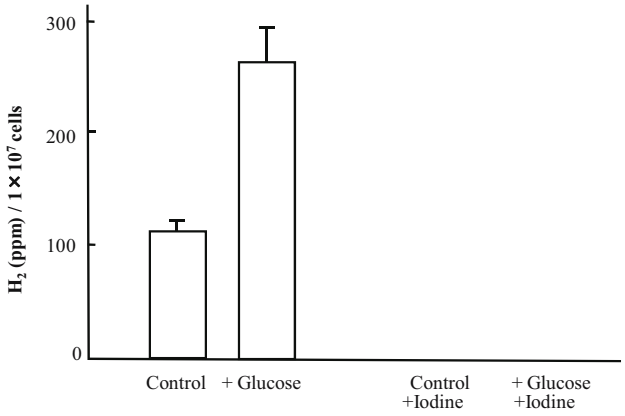


Fig. 5. H_2 generation by *K. pneumonia*. *K. pneumoniae* was cultured overnight in 5 ml BHI medium (pH 7.7) at 37°C. The cultured cells (OD=0.1 at 660 nm) were inoculated into 25 ml BHI medium in the presence or absence of 2% glucose and cultured with shaking at 37°C for 3 h (1×10^7 cells/ml, log phase). H_2 generation was measured in the presence or absence of 100 μM povidone iodine. Data are shown as the Means±SD derived from three separate experiments.

from six healthy volunteers and amplified using primers for Klebsiella 16S rRNA. DNA specific for *K. pneumoniae* was detected in the salivary samples from all volunteers (Fig. 3). Since hydrogenase is the critical enzyme that generates H_2, salivary DNA was also used to amplify hydrogenase using *K. pneumoniae*-specific primers. Figure 4 shows that the gene encoding hydrogenase was also detected in all salivary samples.

Characterization of H_2 generation by *K. pneumoniae*

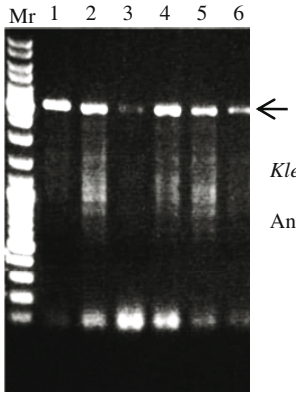
The properties of H_2 generation by *K. pneumoniae* were analyzed quantitatively (Fig. 5). Under standard culture conditions in BHI medium at 37°C, *K. pneumoniae* generated approximately 24 ppm H_2/min/10^7cells. The rate of H_2 generation was enhanced about 2.5-fold by the presence of glucose. In contrast, the rate of H_2 generation was decreased 1/10 by the addition of povidone iodine to the medium. The presence of 3 mM KCN also significantly decreased H_2 generation (Fig. 6).

Effect of hydroxyl radicals on the growth of *K. pneumoniae*

Hydroxyl radicals generated by the Fe-catalyzed Fenton reaction were examined for an effect on the growth of *K. pneumoniae* (Fig. 7). Since KCN inhibits the activity of hydrogenase, the hydroxyl radicals were analyzed in the presence and absence of KCN. Although the growth of *K. pneumoniae* was only slightly suppressed by either hydroxyl radicals or KCN, it was significantly inhibited (by about 50%) by the presence of both.

Oxidative modification of *K. pneumoniae* proteins

The number of protein carbonyls in *K. pneumoniae* increases under standard culture conditions. Thus, the effect of H_2 generation on the occurrence of carbonyl proteins in *K. pneumoniae* was tested (Fig. 8). The number of carbonyl proteins increased following exposure to hydroxyl radicals. Proteins with a molecular weight of 50 kDa and 60-64 kDa



Klebsiella Hydrogenase
Sense: GGTCAACACTACCTTGCG
Antisense: ACTGAACACCACCGTCTC

Fig. 4. Detection of *K. peumoniae* hydrogenase. Each DNA sample from 1 ml of saliva from six healthy volunteers was purified and used to amplify *K. peumoniae* hydrogenase. The PCR products were subjected to 2% agarose gel electrophoresis as described for Fig. 3.

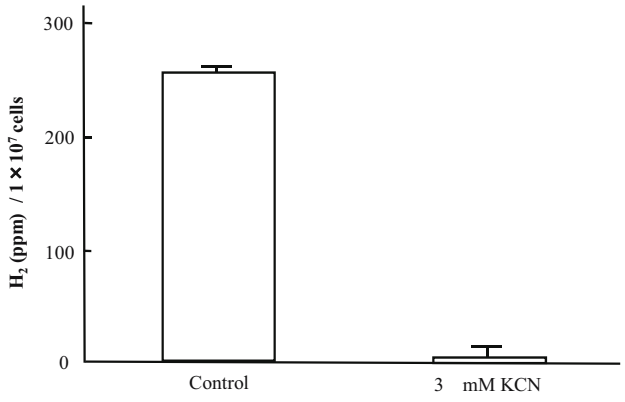


Fig. 6. Effect of KCN on H_2 generation by *K. pneumonia*. *K. pneumoniae* was cultured in 5 ml BHI medium in the presence or absence of 3 mM cyanide (KCN) with shaking at 37°C for 2 h (log phase). Data are shown as the Means±SD derived from three separate experiments.

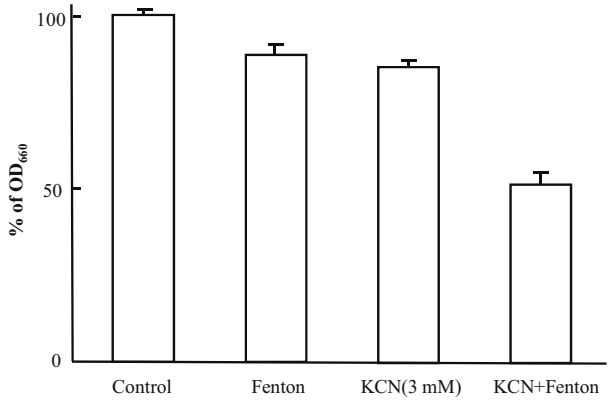


Fig. 7. Effect of oxidative stress on the growth of *K. pneumonia*. *K. pneumoniae* was cultured in 5 ml BHI medium in the presence or absence of 3 mM cyanide (KCN) with shaking at 37°C for 2 h (log phase). H_2O_2 and $FeCl_2$ were added, to a final concentration of 100 μM of each, to elicit a Fenton reaction. Cells were washed and cultured in flesh BHI medium for 1 h. Data are shown as the Means±SD derived from three separate experiments.

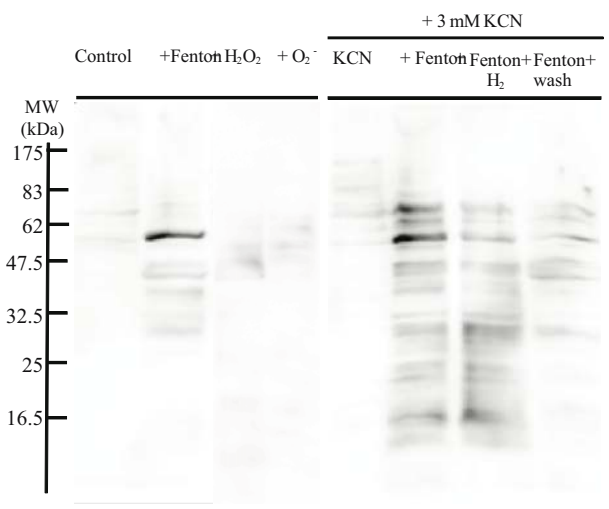


Fig. 8. Oxidative modification of proteins in *K. pneumonia*. *K. pneumoniae* was cultured for 1 h in the presence or absence (control) of either hydroxyl radicals (+ Fenton) or 100 μM of each, to elicit a Fenton reaction. Cells were washed and 100 μM H_2O_2 and superoxide (1 mM hypoxanthine+1 mU xanthine oxidase). The Fenton reaction was elicited by the presence of 100 μM $FeCl_2$ and H_2O_2. Cells were also cultured in the presence of reactive oxygen species and 3 mM KCN and/or 1.9 μM H_2 (+Fenton+H_2). In some experiments, cells exposed to hydroxyl radicals and KCN were washed with the standard medium and subsequently cultured for another 2 h in the same medium (Fenton+wash). Cellular proteins were extracted with 2% SDS, incubated with 8 mM 2,4-dinitrophenylhydrazine at 15°C for 1 h and electrophoresed on SDS-PAGE. Protein carbonyls were reacted with anti-DNP antibody and an HRP-conjugated second antibody as described in the text. The data show one typical example from three separate experiments.

were found to be the major cellular components subjected to oxidative modification. In contrast, the number of carbonyl proteins was not elevated by the presence of either H_2O_2 or O_2. Although KCN alone had no appreciable effect on the number of carbonyl proteins, they increased markedly in the presence of hydroxyl radicals by a mechanism that could be suppressed by H_2. The effect of protein carbonyls on cell viability was tested by washing them with fresh medium and culturing for 1 h in the absence of reactive oxygen species and KCN. The number of carbonylated proteins in the washed and cultured cells markedly decreased.

Discussion

This work revealed the presence of significant H_2 generation in the oral cavity of healthy human volunteers. Orally detectable gases are known to originate predominantly from the respiratory tract and/or pulmonary circulation (Stephensen *et al.*, 1987). However, in this case, oral generation of H_2 was observed under non-expirating conditions, was instantaneously enhanced by the presence of glucose, and was inhibited either by teeth brushing or oral sterilization with povidone iodine. These observations suggested that oral bacteria are responsible for the generation of H_2.

The oral cavity contains more than 300 different species of aerobic, biaerobic and anaerobic bacteria including *S. mutans*, *Streptococcus sanguis*, *K. pneumoniae*, *S. aureus*, *S. epidermidis*, *E. coli*, and *H. influenzae* (Smith *et al.*, 1993; Ohara-Nemoto *et al.*, 2008; Sato *et al*., 2008; Zhu *et al.*, 2008). Among the various bacteria tested, *K. pneumoniae*, *S. aureus* and *S. epidermidis* generated significant amounts of H_2. Hydrogenases are candidate enzymes as the source of H_2 production. These enzymes utilize Ni/Fe- and Fe/Fe-containing isoforms (Adams, 1990; Volbeda *et al.*, 1995). Glucose is metabolized in H_2-generating bacteria via a classic Embden-Meyerhof pathway to generate NADH and the reduced form of ferredoxin (Schut and Adams, 2009). The oxidation of these electron carriers is coupled to the generation of H_2 (Schut and Adams, 2009). Since *K. pneumoniae* contains a $NADP^+$-dependent Ni/Fe-type hydrogenase (Liu and Fang, 2007; Schut and Adams, 2009), this enzyme is the most likely source of H_2 in *K. pneumoniae*. Glucose also enhanced the generation of H_2 in both the oral cavity and in cultured *K. pneumoniae* cells. Among the bacteria examined, *K. pneumoniae* showed the highest H_2-generating activity. The inhibition of H_2 generation by KCN in *K. pneumoniae* supports the premise that the hydroxygenase is principally responsible for the oral synthesis of H_2.

Although the metabolic pathway to generate and use H_2 in anaerobic bacteria has been well documented (Adams, 1990; Volbeda *et al.*, 1995; Adams and Stiefel, 1998; Peters *et al.*, 1998; Steuber *et al.*, 1999; Lyon *et al.*, 2004; Fang *et al.*, 2006; De Lacey *et al.*, 2007), the physiological significance of this stable gas in mammals remains unknown. Hydrogen has been utilized to catalyze hydrogenation of double bonds in a variety of molecules, including unsaturated fatty acids (Lee *et al.*, 2007). Oxidative stress enhances peroxidation of polyunsaturated fatty acids to generate cytotoxic metabolites (Harfoot, 1978). In this context, we previously reported that activated neutrophils infiltrate the oral cavity of healthy human subjects and constitutively generate superoxide and

NO to generate oxidative stress (Sato *et al.*, 2008). The oxidative stress caused by ROS is important for the elimination of pathogens from the oral cavity. In fact, both oral bacteria and the infiltrating neutrophils exhibit significant amounts of oxidized proteins. The presence of this oxidative environment in the oral cavity means that not only pathogens, but also commensal bacteria, should have evolved antioxidant defense systems (Park *et al.*, 2004; Sato *et al.*, 2008; Nishikawa *et al.*, 2009). In this study, both H_2 generation and the growth of *K. pneumoniae* were strongly inhibited by the presence of KCN and $FeCl_2$ plus H_2O_2, a hydroxyl radical generating system. Thus, *de novo* synthesized H_2 might play important roles in the survival and growth of *K. pneumoniae* in oxidative environments.

The mechanism by which H_2 protects *K. pneumoniae* in oxidative environments is not known. In this context, we previously reported that various pathogens, including *H. pylori,* synthesize significant amounts of superoxide radicals and generate protein carbonyls under oxidative conditions similar to those in the oral cavity and gastrointestinal tract (Nagata *et al.*, 1998; Nakahara *et al.*, 1998; Nakamura *et al.*, 2000; Sato *et al.*, 2003; Park *et al*., 2004; Choudhury *et al.*, 2007). Here, the generation of hydroxyl radicals increased the number of protein carbonyls in *K. pneumoniae*, particularly in the presence of KCN, suggesting that H_2 function in the suppression of oxidative injury. It should be noted that the growth of *K. pneumoniae* recovered to control levels after the cells were washed and cultured in a KCN-free standard medium, with concomitant disappearance of the carbonylated proteins. Thus, the proteins carbonylated under oxidative stress might be a key to the survival and growth of *K. pneumoniae*.

The properties of oxidized proteins were analyzed to elucidate the mechanism by which H_2 protects the growth of *K. pneumoniae*. Among the proteins detected, a protein with a molecular weight of 64 kDa was found to be the major band that is oxidatively modified in the absence of H_2. A proline/alanine-rich linker protein that binds to the α subunit of the membranous oxaloacetate decarboxylase/Na^+ pump complex acts as a protector for the transporter (Brondz and Olsen, 1986). This membranous protein is responsible for the transport of Na^+, a prerequisite reaction for the survival of microorganisms. The molecular weight of the major protein band (64 kDa) carbonylated in *K. pneumoniae* is similar to that of the α subunit of the transporter. Thus, oxidation of this membranous protein might be responsible, at least in part, for the inhibition of cell growth. Identification and characterization of the carbonylated proteins in *K. pneumoniae* require further study.

Reperfusion injury of the brain and heart is inhibited by a fairly low concentration of H_2 (Fukuda *et al.*, 2007; Ohsawa *et al.*, 2007; Hayashida *et al.*, 2008; Ohsawa *et al.*, 2008; Nagata *et al.*, 2009). Since H_2 reacts with the hydroxyl radical *in vitro*, scavenging of this radical by H_2 has been postulated as the underlying the mechanism for the inhibition of oxidative tissue injury. However, the reactivity of the hydroxyl radical with biological molecules is extremely high (diffusion-limited reactions) and, hence, non-physiologically high concentrations of scavengers would be required to eliminate the toxic effects of this radical. Thus, it is practically difficult to scavenge the hydroxyl radical directly with physiologically low concentrations of H_2 under *in vivo* conditions. The physiological significance of H_2 generation by oral bacteria, including *K. pneumoniae*, and the protective mechanism of H_2 *in vivo* should be clarified with further studies.

Acknowledgements

This work was supported by grants from the Ministry of Education, Science, Technology and Culture of Japan and Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government.

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