Mechanism of oxygen tolerance in lactic acid bacteria —Function of peroxidase and new iron binding protein, Dpr in oxidative stress—

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Bacteria living in air rely on defense systems that detoxify reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radical which are generated from incomplete reduction of oxygen by enzymatic and nonenzymatic means. These defense systems include (i) enzymes that scavenge reactive oxygen such as superoxide dismutases (SOD), catalases, and peroxidases, (ii) DNA repair enzymes such as exonuclease III, DNA polymerase, and RecA, (iii) protein repair systems such as thioredoxin and methionine sulfoxide reductase, and (iv) proteins which regulate the cellular metabolism of iron to ameliorate the generation of reactive oxygen species.

Lactic acid bacteria, including *Streptococcus mutans*, cannot synthesize heme, and therefore lack catalase and cytochrome oxidases required for energy-linked oxygen metabolism. The growth of lactic acid bacteria, therefore depends strictly on fermentation. Accordingly, the lactic acid bacteria are considered to have a preference for anaerobiosis. However, many lactic acid bacteria can grow in the presence of oxygen, and even consume molecular oxygen through action of flavoenzymes such as NADH oxidase, pyruvate oxidase, and α-glycerophosphate oxidase. Several antioxidant enzymes including manganese superoxide dismutase and none-heme peroxidases such as manganese containing catalase and NADH peroxidase, which may function as substitutes for catalase, were identified and characterized in lactic acid bacteria. Previously, we identified two components of an NADH-dependent peroxidase (AhpC and Nox-1) from *S. mutans*. While studying an *ahpC* and *nox-1* double-disruption mutant of *S. mutans*, we found that the mutant still showed the same level of peroxide tolerance as did the wild-type strain, suggesting the existence of one or more other antioxidant(s) in *S. mutans*. In the preceding paper, we identified a *dpr* gene [Dps (DNA binding protein from starved cells) like peroxide resistance gene] as a potential peroxide resistance gene from chromosomal DNA of *S. mutans* and demonstrated a functional significance for the gene against oxidative stress. The *dpr* disruption mutant of *S. mutans* could not form colonies on agar plates under air. In addition, although *dpr* disruption alone did not interfere with growth in liquid cultures, neither the Δ*dpr*Δ*ahpC*Δ*nox-1* triple mutant nor the Δ*dpr* Δ*sod* double mutant of *S. mutans* was able to grow aerobically in liquid medium. The synthesis of the 20-kDa *dpr* gene product Dpr was found to be induced by exposure of *S. mutans* cells to air. Analysis of deduced primary and secondary structures of Dpr suggested that Dpr is a member of the Dps family of proteins. Dps is a nonspecific DNA-binding protein which accumulates in stationary-phase cells of *Escherichia coli*. The Dps family of proteins form spherical complexes like ferritin which are composed of 7–12 identical subunits of 16–22 kDa and some of them bind iron. To date, three family members including *E. coli* Dps were shown to bind to DNA for protection from oxidative stress. On the other hand, functional divergence of other Dps family proteins was also reported, i.e., the non-heme ferritin of *Listeria innocua*, the fine tangled pilus major subunit of *Haemophilus ducreyi*, the neutrophil activating protein (HP-NAP) of *Helicobacter pylori*, and cold shock protein from *Listeria monocytogenes*. In this study, we purified Dpr from *S. mutans* and examined its molecular properties to understand how Dpr confers oxygen tolerance on *S. mutans*.

Molecular weight determinations of Dpr in solution by analytical ultracentrifugation and light-scattering analyses gave values of 223,000 to 292,000, consistent with a subunit composition of 11.5 to 15 subunits per molecule. The purified Dpr contained iron and zinc atoms and had an ability to incorporate up to 480 iron and 11.2 zinc atoms per molecule. Unlike *E. coli* Dps and two other members of the Dps family, Dpr was unable to bind DNA and did not exhibit catalase activity as reported for *Synechococcus* sp. DpsA. One hundred nM Dpr prevented by more than 90% the formation of hydroxyl radical generated by 10 μM iron(II) salt *in vitro*. Thus, a role in protection against oxidative stress by Dpr might involve the sequestration of iron, thereby preventing the generation of highly toxic radicals and conferring oxygen tolerance. As shown in our experiments, Dpr prevented hydroxyl radical formation generated by 10 μM of iron(II) salt *in vitro*. This result supported our previous suggestion from the *in vivo* study that the failure of a series of Dpr defective mutants to grow under aerobic conditions is caused by the excessive generation of hydroxyl radicals. The high intracellular concentration of Dpr determined by using western blotting (~2% of total cellular soluble protein) might enable cells to be protected from iron-mediated oxidative stress. The contribution of the sequestration of iron by bacterial ferritin to oxidative stress protection was demonstrated in the cases of *Campylobacter jejuni* and of an *E. coli fur recA* mutant. Recently, intracellular "free iron" concentrations of *E. coli* and *Saccharomyces cerevisiae* were reported to be 10.0 and 12.8 μM, respectively, by using whole cell electron paramagnetic resonance analyses. Though the amount of free iron of *S. mutans* is not known, the observed level of Dpr present in *S. mutans* could prevent hydroxyl radical formation for up to 10.0 μM iron(II). In contrast, apo-horse spleen ferritin hardly inhibited hydroxyl radical formation under the conditions used, indicating that under the assay conditions used in this study, there is a competition between nonenzymatic iron oxidation by molecular oxygen [reaction] and iron sequestration by Dpr or horse spleen ferritin.

In bacteria, two types of ferritin molecules, heme-containing bacterioferritin and heme-free ferritin, have been reported. Both are composed of 24 identical or similar subunits. Recent X-ray crystallographic analyses of two members of the Dps family, *E. coli* Dps and *L. innocua* ferritin, revealed them to be spherical dodecamers consisting of four-α-helical bundle monomers, and also delineated their close structural relationship with other bacterial and eucaryotic ferritin proteins. Though the iron binding ability of *E. coli* Dps is not certain, it is clear that *L. innocua* ferritin, *H. pylori* HP-NAP, and *S. mutans* Dpr all incorporate iron. Therefore, at least some members of the Dps protein family including Dpr belong to a new class of small ferritins which appear to exist as dodecamers. Dpr was also able to bind zinc ions. Although a physiological role for the zinc binding ability of Dpr is not known, detoxification of zinc by ferritin during overload has been reported. To date, ferritin-like proteins have not been reported in lactic acid bacteria, and neither bacterioferritin nor none-heme ferritin homologues have been found in genomic sequence data of lactic acid bacteria. In contrast, Dpr and Dps family members were widely distributed among the databases including *S. pyogenes* (AE006586-6), *S. pneumoniae* (AF055720-1), *S. thermophilus*, *S. suis* (AF319974-1), *S. gordonii* (sgord_bvs_203), *E. faecalis* (gef_11370), *L. lactis* (AE006432), *L. rhamnosus* (AF037091-2), and *L. fermentum* (AB049598-1). Taken together with our results from *S. mutans*, Dps family proteins as well as Dpr in lactic acid bacteria may act as ferritin in lactic acid bacteria, and contribute to the ability of these catalase and heme-peroxidase deficient bacteria to grow under air by limiting the iron-catalyzed Fenton reaction.