Review

On the origin of reactive oxygen species and antioxidative mechanisms in *Enterococcus faecalis*

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Enterococci cause serious infections due to a number of virulence factors and wide-spread antibiotic resistance. A molecular mechanism involved in the pathogenesis of enterococcal infections is oxidative stress. *Enterococcus faecalis* produces a variety of antioxidative enzymes involved in the oxidative stress response, a process that is regulated by several transcriptional regulators. In addition, direct production of free radicals derived from oxygen has been proved and hypothesized, respectively, to contribute to the pathogenesis of colorectal cancer and periodontitis. The understanding of molecular mechanisms behind the production of free radicals and the antioxidative status in *E. faecalis* might suggest new alternatives for the treatment of enterococcal infections and related diseases.

Keywords: *Enterococcus faecalis*, reactive oxygen species, oxidative stress, free radicals, infection

Introduction

Enterococci are part of the physiological gastrointestinal flora and are able to survive under harsh conditions (wide range of temperatures, salinity and pH).[1–3] They can act as opportunistic pathogens and are among the leading causes of nosocomial infections.[4] Strains of *Enterococcus faecalis* exhibit a wide, rapidly spreading and growing spectrum of antibiotic resistance.[5] The reason lies in horizontal gene transfer. A genomic analysis of a vancomycin-resistant strain of *E. faecalis* revealed that a considerable part of the genome is made up of foreign sequences, including mobile transposons containing the resistance genes.[6]

E. faecalis represents an important clinical problem due to a number of virulence factors. Although the virulence factors of these opportunistic pathogens have been studied thoroughly in the past, new factors are being uncovered, such as the recently described *ers ace* pathway, which is important in the ability of some strains to colonize the urinary tract.[7] In addition, the spectrum of known virulence factors is widening by research uncovering their novel functions – like the regulation of the oxidative stress response by transcriptional regulation by Ers.[8,9] The same transcription factor has even been found to regulate the expression of genes related to the metabolism of glycerol[10] or citrate.[11] The link between diverse functions of the regulator remains still to be elucidated. The

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Received 14 May 2010, manuscript accepted 6 August 2010

DOI 10.1179/135100010X12826446921581

production of reactive oxygen species and the ability of bacteria to cope with them are important virulence factors in many bacteria, including *E. faecalis*.[12,13]

Oxidative stress is defined as an imbalance between the production of free radicals and antioxidative mechanisms. Increased production of free radicals or decreased antioxidative status leads to oxidative damage of macromolecules including lipids, proteins and DNA. Oxidative stress is the main patho-mechanism in a number of diseases, but its role is clearly established in inflammation.[14] Neutrophils and macrophages are able to produce reactive oxygen species enzymatically, thereby inducing oxidative damage of bacteria. However, their effects are not restricted to pathogens, but may also involve damage to the surrounding host tissue.[15]

Production of free radicals

E. faecalis has been shown to produce superoxide and hydrogen peroxide directly. The role of the production of reactive oxygen species in the pathomechanism of enterococcal infections is unknown. It might be directed against other bacteria colonizing the same niche or against host cells. The gene encoding NADH oxidase (*nox*) is present in the genome of *E. faecalis*.[6] Lactic acid bacteria posses two forms of this enzyme – one produces water and another produces hydrogen peroxide, the latter inhibiting bacterial growth under aerobic conditions *in vitro*.[16] It has been shown that hydrogen peroxide can be produced in glycerol metabolism.[17] According to the study, one of two glycerol metabolism pathways leads to production of hydrogen peroxide. The selection of the pathway has a strain-dependent pattern. Some *E. faecalis* strains produce hydrogen peroxide when the pathway with glycerol-3-phosphate oxidase utilizing oxygen is used. Other strains never use this pathway and, thus, do not produce hydrogen peroxide in glycerol metabolism.

E. faecalis produces superoxide, the main free radical derived from oxygen, via autoxidation of demethylmenaquinone.[18,19] *E. faecalis* is also able to produce hydroxyl radical, the most dangerous free radical *in vivo*, via aromatic hydroxylation.[20] The production of these reactive oxygen species is important for the survival of *E. faecalis in vivo*,[21] but might also induce oxidative damage to DNA of surrounding eukaryotic cells leading to clinically important mutations.[22]

The potential contribution of *E. faecalis* to the pathogenesis of sporadic colorectal cancer has been hypothesized recently,[23] but proof *in vivo* is lacking. The metabolism of *E. faecalis* affects the expression of genes related to apoptosis and cell cycle in the colonic mucosa.[24] A large clinical study showed no association between colonization of the gut with *E. faecalis* and colorectal adenomas or cancer.[25] Interestingly, in a carefully designed study analyzing the bacterial flora in feces, patients suffering from colorectal cancer had higher populations of *E. faecalis*.[26] Whether this finding points to a potential cause or to a consequence of the disease is currently not clear. However, it seems that *E. faecalis* and its metabolism of reactive oxygen species might be involved in the recently proposed CHIEF pathway of colorectal carcinogenesis.[27]

Antioxidative mechanisms

Antioxidative mechanisms enable *E. faecalis* to survive in phagocytes and to colonize extra-intestinal tissues.[12] Antioxidative mechanisms of bacteria confer protection against hydrogen peroxide, hydroxyl radicals and superoxide. The defense against other reactive oxygen or nitrogen species is indirect or non-enzymatic. A number of antioxidative enzymes have been identified in *E. faecalis*.

The oldest and evolutionarily highly conserved enzymes catalyze the dismutation of superoxide. In *E. faecalis*, the importance in virulence has been proved for the manganese-containing superoxide dismutase (MnSOD). Impaired survival of *sodA* mutants challenged by increased production of free radicals under aerobic and anaerobic conditions, and also *in vivo* in a model of murine peritonitis, clearly showed that MnSOD contributes to the virulence of *E. faecalis*.[28] The *sodA* gene is highly divergent but present in most enterococcal strains. The functional importance of *sodA* has been shown recently in tolerance to vancomycin and penicillin.[29]

The ability to express catalase is a virulence factor in some pathogenic bacteria. Although Enterococci are described as catalase-negative,[30] this is not correct in general. Catalase detoxifying hydrogen peroxide was described in *E. faecalis* cells, but the production of active protein is heme–dependent. It requires exogenous heme as prosthetic group to be efficient[31] as *E. faecalis* lacks heme synthesis. This finding might be important for the treatment of enterococcal infections, as some strains can be insensitive to hydrogen peroxide due to active catalase.

A major eukaryotic intracellular antioxidant is glutathione. It acts as a scavenger of free radicals. The oxidized form of glutathione can be reduced enzymatically by glutathione reductase present in

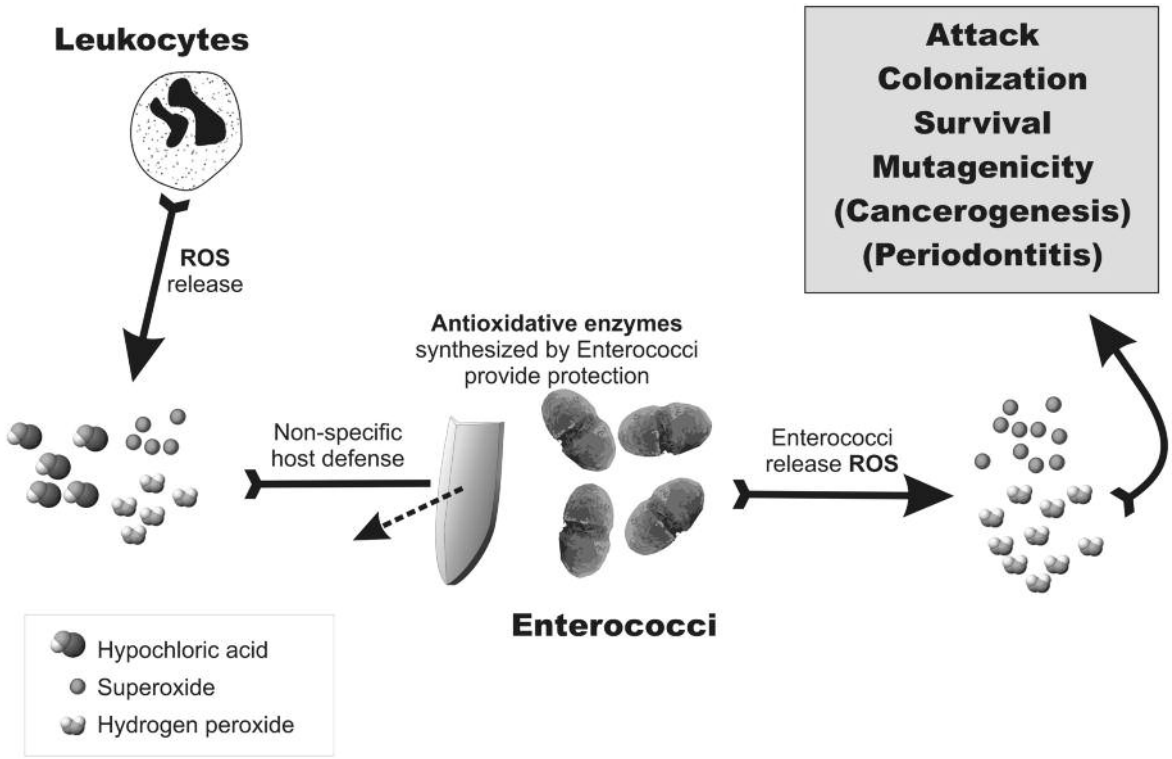

Figure 1 ROS and Enterococci. Enterococci are able to survive oxidative conditions induced by leukocytes like neutrophils and macrophages by induction of antioxidative enzymes. On the other hand, Enterococci directly produce ROS improving their chance of colonization of various niches and survival in comparison to other microbiota. In addition, the enterococcal production of ROS might contribute to the pathogenesis of colon cancer and periodontitis

most eukaryotic cells. The enzyme has been found in several Gram-negative prokaryotes, but also in *E. faecalis*. Interestingly, increased oxygen resulted in up-regulation of glutathione reductase, but not glutathione synthesizing enzymes.[32]

In some pathogenic strains of *E. faecalis*, a pathogenicity island was detected containing a regulator named PerA. Although its function is far from clear, phenotypic analysis of PerA mutants indicated a lower ability to form biofilms and to survive inside macrophages *in vitro* and *in vivo*.[33] The peroxide regulator (PerR), a manganese- and iron-dependent regulator,[34] is a key factor in the response to oxidative stress in some Gram-positive bacteria, but analysis in *E. faecalis* showed a different function. Under oxidative conditions, *E. faecalis* deficient in PerR showed higher survival than wild-type cells. In contrast, peritoneal infection with the *perR* mutant resulted in lower mortality compared to infection with wild-type *E. faecalis* in an animal experiment.[35] This study has shown that PerR is an important virulence factor, but its function in regulating oxidative stress related genes in *E. faecalis* is different from other prokaryotes.

The OxyR regulator, known for its role in the regulation of antioxidant status in *Escherichia coli*, is absent in *E. faecalis*.[13] Interestingly, overexpression of OxyR in *E. faecalis* affects the expression of NADH peroxidase, suggesting a role of OxyR or its analogues in the regulation of antioxidant status in *E. faecalis*.[36] This might be of interest, as an important study designed to analyze the functions of OxyR in *E. coli* showed that OxyR mutants were outperformed by wild-type bacteria in colonization of urinary tract of mice in an *in vivo* experiment.[37] Even more interesting was a negative finding of that study showing that the OxyR is probably not important in the defense against phagocytes as proved in *phox* null mice. Proof for a role of OxyR-related regulation in *E. faecalis* infections is still missing. Alkyl hydroperoxide reductase, thiol peroxidase and other peroxidases are also involved in the metabolism of hydrogen peroxide in *E. faecalis in vitro* and *in vivo*.[38] However, their regulation is far from being clear.

Mutant screening identified a locus in the genome of *E. faecalis* that encodes another oxidative stress regulator in *E. faecalis*.[39] As part of the response to hydrogen peroxide, the designated hydrogen peroxide

regulator (HypR) has been shown to regulate the expression of alkyl hydroperoxide reductase and thiol peroxidase, contributing to the antioxidative status and, thus, to the virulence of *E. faecalis*.[38,40] A later study identified further antioxidant genes regulated by HypR using real-time PCR. These include the most potent enzymatic antioxidants like superoxide dismutase, catalase and glutathione peroxidase, shown to be transcriptionally regulated by the HypR regulator.[41] Indeed, impaired survival of *E. faecalis* in macrophages and improved survival of mice after peritoneal infection has been demonstrated for *hypR* mutants.[40,41]

Conclusions

Oxidative stress seems to play an important role in the pathogenesis of infections with virulent strains of *E. faecalis*. In addition, reactive oxygen species produced directly by *E. faecalis* might contribute to the pathogenesis of colorectal cancer and periodontitis.[23,42] Recently, *E. faecalis* has been hypothesized to be a candidate for the origin of salivary markers of oxidative stress.[43] The therapeutic deficit against strains of *E. faecalis* resistant to a wide spectrum of antibiotics requires research into new potential interventions.[44] As the molecular mechanisms behind the antioxidative status as well as the production of free radicals are known in detail (Fig. 1), new therapeutic and/or preventive alternatives against enterococcal infections might arise in the near future.

Acknowledgemant

This publication is the result of the project implementation: *'Centre of excellence for exploitation of informational biomacromolecules in the disease prevention and improvement of quaity of life'* supported by thr Research & Development Operational Programme funded by thr ERDF.

References

1. Gardini F, Martuscelli M, Caruso MC *et al*. Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*. *Int J Food Microbiol* 2001; **64**: 105–117.
2. Van den Berghe E, De Winter T, De Vuyst L. Enterocin A production by *Enterococcus faecium* FAIR-E 406 is characterised by a temperature- and pH-dependent switch-off mechanism when growth is limited due to nutrient depletion. *Int J Food Microbiol* 2006; **107**: 159–170.
3. Moreno MRF, Sarantinopoulos P, Tsakalidou E *et al*. The role and application of enterococci in food and health. *Int J Food Microbiol* 2006; **106**: 1–24.
4. Jett BD, Huycke MM, Gilmore MS. Virulence of Enterococci. *Clin Microbiol Rev* 1994; **7**: 462–467.
5. Gonzales RD, Schreckenberger PC, Graham MB *et al*. Infections due to vancomycin-resistant *Enterococcus faecium* resistant to linezolid. *Lancet* 2001; **357**: 1179.
6. Paulsen IT, Banerjei L, Myers GSA *et al*. Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* 2003; **299**: 2071–2074.
7. Lebreton F, Riboulet-Bisson E, Serror P *et al*. *ace*, which encodes an adhesin in *Enterococcus faecalis*, is regulated by Ers and is involved in virulence. *Infect Immun* 2009; **77**: 2832–2839.
8. Giard JC, Riboulet E, Verneuil N *et al*. Characterization of Ers, a PrfA-like regulator of *Enterococcus faecalis*. *FEMS Immunol Med Microbiol* 2006; **46**: 410–418.
9. Riboulet-Bisson E, Le Jeune A, Benachour A *et al*. Ers a Crp/Fnr-like transcriptional regulator of *Enterococcus faecalis*. 15th Meeting of the Lactic-Acid-Bacteria-Club. Rennes, France, 2007; 71–74.
10. Riboulet-Bisson E, Hartke A, Auffray Y *et al*. Ers controls glycerol metabolism in *Enterococcus faecalis*. *Curr Microbiol* 2009; **58**: 201–204.
11. Riboulet-Bisson E, Sanguinetti M, Budin-Verneuil A *et al*. Characterization of the Ers regulon of *Enterococcus faecalis*. *Infect Immun* 2008; **76**: 3064–3074.
12. Riboulet E, Verneuil N, La Carbona S *et al*. Relationships between oxidative stress response and virulence in *Enterococcus faecalis*. *J Mol Microbiol Biotechnol* 2007; **13**: 140–146.
13. Storz G, Imlay JA. Oxidative stress. *Curr Opin Microbiol* 1999; **2**: 188–194.
14. Smith JA. Neutrophils, host-defense and inflammation – a double-edged sword. *J Leukoc Biol* 1994; **56**: 672–686.
15. Libby P. Inflammatory mechanisms: the molecular basis of inflammation and disease. *Nutr Rev* 2007; **65**: S140–S146.
16. Sakamoto M, Komagata K. Aerobic growth of and activities of NADH oxidase and NADH peroxidase in lactic acid bacteria. *J Ferment Bioeng* 1996; **82**: 210–216.
17. Bizzini A, Zhao C, Budin-Verneuil A *et al*. Glycerol is metabolized in a complex and strain dependent manner in *Enterococcus faecalis*. *J Bacteriol* 2010; **192**: 779–785.
18. Huycke MM, Abrams V, Moore DR. *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. *Carcinogenesis* 2002; **23**: 529–536.
19. Huycke MM, Moore D, Joyce W *et al*. Extracellular superoxide production by *Enterococcus faecalis* requires demethylmenaquinone and is attenuated by functional terminal quinol oxidases. *Mol Microbiol* 2001; **42**: 729–740.
20. Huycke MM, Moore DR. *In vivo* production of hydroxyl radical by *Enterococcus faecalis* colonizing the intestinal tract using aromatic hydroxylation. *Free Radic Biol Med* 2002; **33**: 818–826.
21. Huycke MM, Gilmore MS. *In vivo* survival of *Enterococcus faecalis* is enhanced by extracellular superoxide production. In: Horaud T, Bouvet A, Leclercq R *et al*. (eds) XIII Lancefield International Symposium on Streptococci and Streptococcal Diseases. Paris, France: Plenum Press Div Plenum Publishing Corp 1996; 781–784.
22. Wang XM, Huycke MM. Extracellular superoxide production by Enterococcus faecalis promotes chromosomal instability in mammalian cells. *Gastroenterology* 2007; **132**: 551–561.
23. Wang XM, Allen TD, May RJ *et al*. *Enterococcus faecalis* induces aneuploidy and tetraploidy in colonic epithelial cells through a bystander effect. *Cancer Res* 2008; **68**: 9909–9917.
24. Allen TD, Moore DR, Wang XM *et al*. Dichotomous metabolism of *Enterococcus faecalis* induced by haematin starvation modulates colonic gene expression. *J Med Microbiol* 2008; **57**: 1193–1204.
25. Winters MD, Schlinke TL, Joyce WA *et al*. Prospective case-cohort study of intestinal colonization with enterococci that produce extracellular superoxide and the risk for colorectal adenomas or cancer. *Am J Gastroenterol* 1998; **93**: 2491–2500.
26. Balamurugan R, Rajendiran E, George S *et al*. Real-time polymerase chain reaction quantification of specific butyrate-producing bacteria, *Desulfovibrio* and *Enterococcus faecalis* in the feces of patients with colorectal cancer. *J Gastroenterol Hepatol* 2008; **23**: 1298–1303.
27. Slattery ML, Fitzpatrick FA. Convergence of hormones, inflammation, and energy-related factors: a novel pathway of cancer etiology. *Cancer Prev Res* 2009; **2**: 922–930.
28. Verneuil N, Maze A, Sanguinetti M *et al*. Implication of

(Mn)superoxide dismutase of *Enterococcus faecalis* in oxidative stress responses and survival inside macrophages. *Microbiology* 2006; **152**: 2579–2589.
29. Bizzini A, Zhao C, Auffray Y *et al*. The *Enterococcus faecalis* superoxide dismutase is essential for its tolerance to vancomycin and penicillin. *J Antimicrob Chemother* 2009; **64**: 1196–1202.
30. Fisher K, Phillips C. The ecology, epidemiology and virulence of Enterococcus. *Microbiology* 2009; **155**: 1749–1757.
31. Frankenberg L, Brugna M, Hederstedt L. *Enterococcus faecalis* heme-dependent catalase. 6th International Conference on Streptococcal, Lactococcal, and Enterococcal Genetics. Asheville, North Carolina: Am Soc Microbiol 2002; 6351–6356.
32. Patel MP, Marcinkeviciene J, Blanchard JS. *Enterococcus faecalis* glutathione reductase: purification, characterization and expression under normal and hyperbaric O-2 conditions. *FEMS Microbiol Lett* 1998; **166**: 155–163.
33. Coburn PS, Baghdayan AS, Dolan GT *et al*. An AraC-type transcriptional regulator encoded on the *Enterococcus faecalis* pathogenicity island contributes to pathogenesis and intracellular macrophage survival. *Infect Immun* 2008; **76**: 5668–5676.
34. Herbig AF, Helmann JD. Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. *Mol Microbiol* 2001; **41**: 849–859.
35. Verneuil N, Rince A, Sanguinetti M *et al*. Contribution of a PerR-like regulator to the oxidative-stress response and virulence of *Enterococcus faecalis*. *Microbiology* 2005; **151**: 3997–4004.
36. Ross RP, Claiborne A. Evidence for regulation of the NADH peroxidase gene (*npr*) from *Enterococcus faecalis* by OxyR. *FEMS Microbiol Lett* 1997; **151**: 177–183.
37. Johnson JR, Clabots C, Rosen H. Effect of inactivation of the global oxidative stress regulator oxyR on the colonization ability of *Escherichia coli* O1:K1:H7 in a mouse model of ascending urinary tract infection. *Infect Immun* 2006; **74**: 461–468.
38. La Carbona S, Sauvageot N, Giard JC *et al*. Comparative study of the physiological roles of three peroxidases (NADH peroxidase, alkyl hydroperoxide reductase and thiol peroxidase) in oxidative stress response, survival inside macrophages and virulence of *Enterococcus faecalis*. *Mol Microbiol* 2007; **66**: 1148–1163.
39. Verneuil N, Le Breton Y, Hartke A *et al*. Identification of a new oxidative stress transcriptional regulator in *Enterococcus faecalis*. *Lait* 2004; **84**: 69–76.
40. Verneuil N, Sanguinetti M, Le Breton Y *et al*. Effects of the *Enterococcus faecalis hypR* gene encoding a new transcriptional regulator on oxidative stress response and intracellular survival within macrophages. *Infect Immun* 2004; **72**: 4424–4431.
41. Verneuil N, Rince A, Sanguinetti M *et al*. Implication of *hypR* in the virulence and oxidative stress response of *Enterococcus faecalis*. *FEMS Microbiol Lett* 2005; **252**: 137–141.
42. Kayaoglu G, Orstavik D. Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. *Crit Rev Oral Biol Med* 2004; **15**: 308–320.
43. Vlkova B, Celec P. Does *Enterococcus faecalis* contribute to salivary thiobarbituric acid-reacting substances? *In Vivo* 2009; **23**: 343–345.
44. Tendolkar PM, Baghdayan AS, Shankar N. Pathogenic enterococci: new developments in the 21st century. *Cell Mol Life Sci* 2003; **60**: 2622–2636.