Redox Balance via Lactate Dehydrogenase Is Important for Multiple Stress Resistance and Virulence in Enterococcus faecalis

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Enterococcus faecalis is a highly stress resistant opportunistic pathogen. The intrinsic ruggedness of this bacterium is supposed to be the basis of its capacity to colonize the hostile environments of hospitals and to cause several kinds of infections. We show in this work that general resistance to very different environmental stresses depends on the ability of E. faecalis to maintain redox balance via lactate dehydrogenase (LDH). Furthermore, LDH-deficient mutants are less successful than the wild type at colonizing host organs in a murine model of systemic infection. Taken together, our results, as well as those previously published for Staphylococcus aureus (A. R. Richardson, S. J. Libby, and F. C. Fang, Science 319:1672–1676, 2008), identify LDH as an attractive drug target. These drugs may have additional applications, as in the fight against glycopeptide antibiotic-resistant bacteria and even cancer.
of lactate production in the \(ldh-1\) \(ldh-2\) double mutant virtually eliminated growth, during nitrosative stress (14). The latter study also demonstrated that the virulence of the \(\Delta ldh-1\) mutant was decreased in mice, and the \(\Delta ldh-1\) \(\Delta ldh-2\) mutant was virtually avirulent. The combined results for \(S. aureus\) led to the conclusion that lactate metabolism in general, and especially the NO·-inducible enzyme LDH-1, is crucial for adaptation to nitrosative stress and for resistance to innate immunity in this important human pathogen.

On the basis of these results, we wondered if there was also a link between lactate metabolism, stress responses, and virulence in \(E. faecalis\). We found that LDH-deficient mutants constructed in two different backgrounds are generally more sensitive than wild-type strains to environmental stresses and exhibit attenuated virulence.

### MATERIALS AND METHODS

#### Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in Table 1. The \(E. faecalis\) strains were cultured on M17 medium (15) supplemented with 0.5% (wt/vol) glucose (GM17 medium) at 37°C. Overnight precultures, grown without agitation in 10 ml GM17 broth, were used to inoculate the cultures for growth analysis under both normal and stress conditions. For the acidic pH stress analysis, M17MOPS medium, which contains 200 mM 3-[(N-morpholinio)propanesulfonic acid (MOPS) as a buffer instead of the glycerophosphate in M17 medium, was used. For growth analysis, optical densities at 600 nm (OD\(_{600}\)) were followed on a BioPhotometer (Eppendorf).

#### Construction of \(H2\)-2 \(ldh\) deletion mutants and the complemented strain

Plasmids and PCR products were purified using a Nucleospin plasmid kit and a Nucleospin Extract II kit, respectively (Macherey-Nagel, Düren, Germany). Other standard techniques were carried out as described by Sambrook et al. (16). The \(E. faecalis\) \(H2\)-2 \(\Delta ldh-1\) and \(\Delta ldh-2\) mutants were constructed by deletion of 341-bp and 307-bp internal fragments, respectively. The \(\Delta ldh-1\) \(\Delta ldh-2\) double mutant was constructed by introducing the \(ldh-2\) deletion into the \(\Delta ldh-1\) mutant. \(H2\)-2 mutants were constructed by using the oligonucleotides listed in Table 2 and plasmid pMAD according to the protocol published by La Carbona et al. (17). The construction of the \(E. faecalis\) V583 \(\Delta ldh\) mutants is described in the article of Jönsson and coworkers (2).

The \(ldh1R158E\) mutant was generated by site-directed mutagenesis in a 2-step PCR procedure. First, the \(ldh-1\) gene with upstream and downstream sequences was amplified using primer pairs pMad_\(ldh\_Nco\_for/\)Mut_\(ldh\_rev\) and pMad_\(ldh\_Bam\_rev/Mut_\(ldh\_for\), respectively (Table 2). The overlapping amplimers were then used as templates for the second step using the pMad_\(ldh\_Nco\_for/\)pMad_\(ldh\_Bam\_rev\) primers, and the PCR product was cloned into the pMAD vector as described previously, generating plasmid pdl\(dh\_R158E\) (17). This plasmid was transformed into the \(H2\)-2 \(\Delta ldh-2\) mutant. The replacement of the arginine codon (CGT) with the glutamic acid codon (GAA) was verified by sequencing, and the absence of lactate production in the \(ldh1R158E\) mutant was confirmed by high-performance liquid chromatography (HPLC) (data not shown).

For complementation experiments, the \(ldh-1\) gene, including its ribosome binding site (RBS), was amplified with oligonucleotides \(ldh13535Bam\) and \(ldh13535Sp\) (Table 2) and was cloned into plasmid pMSP3535 (18) under the control of the nisin-inducible promoter of that vector. The plasmid was then transformed into the \(H2\)-2 \(\Delta ldh-1\) \(\Delta ldh-2\) double mutant. Wild-type \(H2\)-2 and the \(\Delta ldh-1\) \(\Delta ldh-2\) double mutant strain, each harboring the empty pMSP3535 plasmid, were used as controls. Where indicated, nisin was added to cultures at an OD\(_{600}\) of 0.2 to a final concentration of 0.5 µg/ml.

#### Growth of \(E. faecalis\) strains under stress conditions

For \(H2O2\), HOCl, SDS, ethanol, and dimethyl sulfoxide (DMSO) stresses, cells were...
grown in GM17 medium until they reached an OD_{600} of 0.2. At this moment, the stressing agent was added. Except for the H_{2}O_{2} experiments, cultures were grown in 30-ml tubes containing 10 ml of medium and were incubated before and after the addition of the stressing agents without agitation in a 37°C water bath. For H_{2}O_{2}, cultures were grown in 150-ml Erlenmeyer flasks filled with 15 ml of medium and were incubated before and after the addition of the peroxide with agitation at 120 rpm in a 37°C incubator. For the NaCl, glucose, and acid stresses, the cultures were grown in GM17 medium to an OD_{600} of 0.2. At this moment, cultures were centrifuged (5,000 × g, 4°C, 10 min), and the cells were taken up in GM17 medium containing 8% NaCl or 10% glucose or in M17MOPS medium with 0.25% glucose adjusted with HCl to pH 4.5. In the serum experiments, the cells were cultured in brain heart infusion (BHI) medium containing 40% filter-sterilized serum, in 30-ml tubes with 10 ml of medium and were incubated without agitation in a 37°C water bath. The final concentrations of the stressing agents were as follows: 2.5 mM H_{2}O_{2}, 0.25% HOCl, 8% ethanol, 0.01% SDS, 10% DMSO, 8% NaCl, and 10% glucose.

Virulence experiments. To test the virulence of both JH2-2 and V583 ldh deletion mutants, we used an adaptation of a well-established intravenous infection model (19), as described by Michaux et al. previously (20). Briefly, the strains were cultured overnight in BHI broth supplemented with 40% heat-inactivated horse serum; the cultures were centrifuged; and the resulting pellets were resuspended in sterile phosphate-buffered saline (PBS). These bacterial suspensions contained 1 × 10^8 bacteria/ml. A 100-μl aliquot from each strain suspension was injected into the tail vein of each of five female BALB/c mice (10 weeks old; Harlan Italy Srl, San Pietro al Natisone, Udine, Italy). Seven days after infection, mice were used for the determination of CFU/g organ. CFU counts resulting from three replicated experiments (for a total of 15 mice per infection group) were analyzed by the unpaired t test.

RESULTS AND DISCUSSION

LDH-deficient mutants are generally stress sensitive. In order to evaluate the metabolic potential of E. faecalis for ethanol production, we recently constructed LDH deletion mutants in strain JH2-2 (3). E. faecalis harbors two separate genes, named ldh-1 and ldh-2, encoding lactate dehydrogenases named LDH-1 and LDH-2. Deletion mutants of each ldh gene, as well as an Δldh-1 Δldh-2 double mutant, were constructed in strain JH2-2. Corresponding LDH deletion mutants of the E. faecalis clinical isolate V583 have also been constructed and characterized recently (2). The results showed that LDH-1 is the main enzyme in lactate production, which we confirmed for strain JH2-2 in our previous study (3). The growth rates of the V583 LDH deletion mutants and of the corresponding wild-type strain were comparable in chemically defined medium (2). This was confirmed and was also found for wild-type V583 and JH2-2 and their ldh deletion mutants in complex GM17 medium (Fig. 1). The final pH values were pH 6.0 for wild-type JH2-2 and the Δldh-1 and Δldh-2 strain and pH 6.2 for the isogenic Δldh-1 mutant and the Δldh-1 Δldh-2 double mutant under these experimental conditions.

LDH mutants of S. aureus have been shown to be sensitive to nitrosative stress (14). We wondered if this would also be the case for enterococci. To test this possibility, cultures of the JH2-2 wild-type strain and the ldh double deletion mutant were exposed to 1 mM NO· donor S-nitroso-N-acetyl-DL-penicillamine (SNAP). The Δldh-1 Δldh-2 double mutant was more sensitive than the wild type to this treatment. However, this difference in survival was also observed with the control compound NAP (N-acetyl-DL-penicillamine), which does not generate NO. Since the compounds were dissolved in DMSO, the only plausible explanation of the results obtained was that Δldh mutants were sensitive to the solvent and not to the NO-generating agent. This was tested by exposing the strains to 10% DMSO (Fig. 2). This experiment showed, indeed, that the growth rate of the JH2-2 Δldh-1 mutant and, even more, that of the JH2-2 Δldh-1 Δldh-2 double mutant were lower than that of the parent strain. In contrast, the Δldh-2 mutant grew as well as the wild-type strain (Fig. 2).

Since an effect of reactive nitrogen species (RNS) on the physiology of the bacterium might have been masked by the stress imposed by the solvent DMSO, we subsequently tested the resistance of the various ldh deletion mutants of both strains (JH2-2 and V583) to reactive oxygen species (ROS). The growth kinetics of wild-type JH2-2 and V583 and their corresponding ldh deletion mutant strains were monitored in the presence of 2.5 mM H_{2}O_{2} (Fig. 3a and b). This treatment provoked an extended growth lag of several hours for all strains. In both backgrounds, Ldh-2-deficient mutants showed little (JH2-2 Δldh-2) or no (V583 Δldh-2) difference in growth recovery from the corresponding parent strains. In contrast, the JH2-2 and V583 Δldh-1 single mutants and Δldh-1 Δldh-2 double mutants were greatly affected by this treatment (Fig. 3a and b). The Δldh-1 mutants escaped from growth inhibition earlier (significant growth after 8 h of incubation).
than the corresponding double mutants. This difference was more pronounced in the V583 background, where the $\Delta ldh$-1 $\Delta ldh$-2 mutant demonstrated no growth even at the end of the experiment. Bringing back the wild-type $ldh$-1 gene on a plasmid under the control of a nisin-inducible promoter into the JH2-2 $\Delta ldh$-1 $\Delta ldh$-2 double mutant restored growth to near-wild-type levels (Fig. 3c), demonstrating that LDH deficiency is responsible for the observed sensitivity to the oxidant.

Since we observed that $ldh$ mutants were more susceptible to such different treatments as a solvent and an oxidant, we wondered if a deficiency in lactate production might have a more general negative effect on stress resistance. Therefore, we tested the growth of the parent strains and the $ldh$-deficient mutants in the presence of various stressing agents. As can be seen from Fig. 2, the growth of the JH2-2 and V583 $\Delta ldh$-1 mutants and, especially, the $\Delta ldh$-1 $\Delta ldh$-2 double mutants was always more affected in the presence of ethanol (8%), the strong oxidant hypochlorite (0.25%), or the detergent SDS (0.01%), at an acidic pH (pH 4.5), or under conditions of high osmolarity (8% NaCl or 10% glucose) than that of their parent strains (see also Fig. S2 in the supplemental material). Except for ethanol, which had greater effects in the JH2-2 background, the effects of the stresses on the growth of the wild-type and mutant strains were very comparable for the JH2-2 and V583 backgrounds. The growth performance of the $\Delta ldh$-2 mutants under most stress conditions was similar to that of the corresponding wild-type strains, with the exception of growth at pH 4.5.

**FIG 2** Growth of wild-type JH2-2 and V583 cells (filled diamonds) and their isogenic $ldh$-deficient mutants (shaded triangles, $\Delta ldh$-2 mutant; shaded squares, $\Delta ldh$-1 mutant; multiplication signs, $\Delta ldh$-1 $\Delta ldh$-2 mutant) in different stressing environments. Only relevant parts of the growth curves are shown for each treatment. EtOH, ethanol; Gluc, glucose. See Fig. S2 in the supplemental material for full growth curves.
these conditions, the Δldh-2 mutant was slightly but reproducibly more susceptible than the parent strain (Fig. 2).

The combined results seem to indicate that the characteristic intrinsic ruggedness of *E. faecalis* under environmental stresses is dependent on the metabolic capacity to reduce pyruvate to lactate by lactate dehydrogenase. However, the possibility that the growth deficiency of *ldh* deletion mutants in stressing environments is related not to the activity of the LDH proteins but simply to their physical disappearance could not be excluded. We therefore constructed another *ldh* double mutant by introducing an *ldh*-1 allele with point mutations in the substrate binding domain (http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=96482), leading to the replacement of the critical arginine at position 158 of the enzyme with glutamate, into the *ldh*-2 mutant (see Materials and Methods for more details). Arginines at this position in LDH interact with the carboxyl group of pyruvate. The replacement of this residue should therefore lead to inactivation of the enzyme, and HPLC analysis of the Δldh/R158E/ldh-2 mutant confirmed the absence of lactate production (data not shown). This new mutant showed a fitness deficiency with regard to growth in stressing environments comparable to that of the Δldh-1 Δldh-2 double mutants, supporting our initial hypothesis that a deficiency in LDH activity is responsible for the elevated stress sensibility (data not shown).

What might be the molecular explanations for the observed multiple stress sensitivities of LDH-deficient mutants? Based on data in the literature, at least two hypotheses, which are not mutually exclusive, could be proposed. In the first, the functioning of the alternative pathways for redox homeostasis in the LDH-deficient strains may be generally more sensitive to perturbations of cell physiology in stressing environments than the maintenance of redox homeostasis via LDH. Some results obtained with *S. aureus* and *E. faecalis* point in this direction. Indeed, the activities at the beginning of these alternative pathways, i.e., pyruvate dehydrogenase (PDH) and pyruvate formate lyase (PFL), are strongly inhibited by NO in extracts of *S. aureus* cells, whereas LDH remains nearly 100% active under these conditions (14). Furthermore, the PFL activity has also been shown to be sensitive to an acidic pH in *E. faecalis* (21). Interestingly, expression of the operons encoding PDH and PFL is induced in the V583 Δldh-1 Δldh-2 double mutant (6). Both PDH and PFL compete with LDH for the substrate pyruvate. PDH generates acetyl coenzyme A (acetyl-CoA) in the following oxidative decarboxylation reaction: pyruvate + NAD$^+$ + CoASH → acetyl-CoA + NADH + CO$_2$ (where CoASH is coenzyme A). PFL converts pyruvate to acetyl-CoA and formate in the following reaction: pyruvate + CoASH → acetyl-CoA + formate. The redox balance can then be achieved by reducing acetyl-CoA to ethanol, which allows the oxidation of 2 NADH molecules to NAD$^+$.

The second explanation could be that lactate formation may trigger a process that induces a general stress resistance response. This hypothesis seems to be supported by results from cancer research. Most solid tumors are characterized by increased glucose uptake and lactate formation (22). Studies on experimental tumors have shown that lactate concentrations are positively correlated with radioresistance (23), and a high intratumor lactate concentration might also be responsible for the chemoresistance of some tumors (24).

In any case, our results clearly demonstrate that the fitness of LDH-deficient strains is significantly reduced in stressing environments. We wondered if this LDH deficiency also influences the virulence of the mutants.

**LDH-deficient mutants exhibit attenuated virulence.** Pathogens invading a host are exposed to numerous stresses, and stress resistance has been correlated with virulence (25). To analyze if LDH deficiency reduces the resistance of *E. faecalis in vivo*, we first tested the growth of the *ldh* deletion mutants and of the parent strains, JH2-2 and V583, in BHI medium containing 40% horse serum (Fig. 2). LDH-2 deficiency had no effect on growth relative to the growth of controls under these conditions. In contrast, the growth of the Δldh-1 mutants was significantly reduced, and growth restriction was even more pronounced in the Δldh-1 Δldh-2 double mutants. Indeed, whereas the wild-type strains reached an OD$_{600}$ of ca. 0.63 after 3 h of growth, the strain deficient in both LDH-1 and LDH-2 showed no significant growth until this time point. Inhibition of the growth of the *ldh* double
deletion mutant was also observed by using serum pretreated at 56°C for 30 min, a treatment generally used to inactivate the complement system. However, the growth of the \( \Delta ldh-1 \Delta ldh-2 \) double mutant was comparable to that of the wild-type strain in BHI medium containing 40% serum treated at 65°C for 30 min, demonstrating that proteins more heat stable than the complement system are involved in this inhibition of the growth of LDH-deficient strains.

Then we assessed whether the single \( \Delta ldh-1 \) or \( \Delta ldh-2 \) mutation or the double \( \Delta ldh-1 \Delta ldh-2 \) mutation could affect the virulence of JH2-2 and V583 by comparing the mutant and wild-type strains in a murine model of systemic infection. Seven days after intravenous bacterial challenge, groups of mice were sacrificed, and their kidneys and livers were removed for the enumeration of viable bacteria. As shown in Fig. 4, the counts (in CFU per gram of tissue) of both JH2-2 (Fig. 4a and b) and V583 (Fig. 4c and d) in the infected organs did not differ significantly between the single mutants and the parent strains. Conversely, the numbers of cells of the \( \Delta ldh-1 \Delta ldh-2 \) double mutants recovered were significantly lower in both the kidneys (\( P, 0.005 \) for JH2-2 and 0.003 for V583) and the liver (\( P, 0.005 \) for JH2-2 and 0.002 for V583) than those of the wild-type strains in both the JH2-2 and V583 backgrounds.
(Fig. 4). These findings indicate that functionally active \( \Delta ldh-1 \) and \( \Delta ldh-2 \) genes contribute together to enterococcal persistence within mouse organs, since only the simultaneous inactivation of both genes greatly reduces the ability of \( E. faecalis \) to colonize and infect the host. From these results, it can also be deduced that the residual activity of LDH-2 in the \( \Delta ldh-1 \) single mutant seems to be sufficient for colonization of the organs. Of note also, strain JH2-2 accumulated to very high numbers in the kidneys compared to strain V583, but the corresponding double mutant did not grow to numbers higher than those of the V583 homologous mutant.

In conclusion, the results presented in this report establish for the first time that the intrinsic ruggedness of \( E. faecalis \), supposed to be important for its persistence in hospital settings and its capacity to cause infections, can be weakened by disabling its capacity to maintain redox balance via the LDH reaction. Furthermore, we showed that this also decreases fitness during infection, arguing that LDH may be an attractive drug target. Such a drug would have additional applications. Glycopeptide antibiotics such as vancomycin inhibit peptidoglycan synthesis by binding to the C-terminal d-alanyl-d-alanine of pentapeptide precursors, preventing transglycosylation and transpeptidation in cell wall assembly. Vancomycin resistance is based on the synthesis of modified peptidoglycan precursors ending in d-alanyl-d-lactate, to which glycopeptides exhibit low binding affinities. D-lactate is synthesized by VanH dehydrogenase, a D-LDH converting pyruvate into D-lactate (26). Inactivation of this enzyme would counteract glycopeptide resistance. Finally, drugs inactivating LDH might also be useful for increasing the sensitivity of tumors to radiation and/or chemotherapy (23, 24).

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