Identification and molecular analysis of superoxide dismutase isoforms in *Helicobacter pylori*

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Abstract

Three electromorphs of iron superoxide dismutase (FeSOD) were identified among 29 *Helicobacter pylori* isolates by native gel electrophoresis and activity staining. The electromorphs designated isoforms A, B, and C are characterized by slow, intermediate and fast electrophoretic migration, respectively, which was not observed under denaturing conditions. The isoforms were not associated with virulence determinants and with the outcome of disease. Sequence analysis of the *sodB* gene in strains producing different FeSOD isoforms and comparison of deduced protein sequences revealed that differences in the electric migration behavior are associated with exchange of charged amino acids, suggesting that faster migration is caused by a more negative total charge of the proteins. Electrophoretic migration of native FeSOD was not influenced by changes in the iron cofactor concentration, oxidative stress, and different media, indicating that FeSOD isoforms represent stable strain-specific markers. ß 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Superoxide dismutases (SODs) catalyze conversion of the superoxide anion to hydrogen peroxide which is dis-integrated to oxygen and water by catalase. These functions detoxify reactive oxygen species and protect cells from lethal oxidative damage [6]. In pathogenic bacteria SODs contribute to virulence and support the ability to face the immune system [4,6,11,12]. The importance of SODs for bacteria is underlined by the fact that some species carry a set of different SODs which are cofactored by various metals like iron, manganese, copper, or zinc, and are regulated by changes in metal cofactor or oxygen concentration [3,4,6].

Investigation of SOD in strains 2012 and 60190 revealed that the gastric pathogen *Helicobacter pylori* produces a single iron-cofactored SOD (FeSOD) [7,9,13]. Because of the possible importance of SOD in infection, it was the aim of this study to investigate FeSOD in additional *H. pylori* strains and clinical isolates from patients with non-ulcer dyspepsia (NUD) and peptic ulcer disease (PUD) to evaluate possible correlations between SOD production and outcome of disease. Analysis by native gel electrophoresis and activity staining revealed that isoforms of *H. pylori* FeSOD exist, which were further studied with respect to analysis of the coding gene and the electrophoretic migration behavior under various environmental conditions.

2. Materials and methods

2.1. Bacterial strains and growth conditions

*H. pylori* was cultivated on HHP blood agar or on Brucella broth agar with 5% fetal calf serum (BBF) at 37°C under microaerobic conditions as described [2]. Growth experiments under conditions of iron overload, iron depletion or oxidative stress were performed on BBF agar with various supplements at sublethal concentrations [2]; iron overload was generated with ferric or ferrous chloride (Sigma F2130, F7134) at concentrations of 0.1 or 1 mM. Iron deprivation and oxidative stress were achieved by adding deferoxamine mesylate (Sigma D9533), and para-
quat (methyl viologen, Sigma M2254), respectively. Stress conditions were verified by a visible growth inhibition (about 50%) as determined earlier [2].

2.2. Analysis of FeSOD by activity staining

Proteins were extracted from bacteria grown for 2 days on agar, and FeSOD was analyzed by native gel electrophoresis and visualized by activity staining as described [13]. Purified FeSOD and MnSOD from Escherichia coli (Sigma S5389 and S5639) served as controls. The relative mobility (Rm) values of the SOD proteins were calculated after electrophoretic separation on a 10% polyacrylamide gel.

2.3. Analysis of the sodB gene

Isolation of DNA, PCR amplification and sequence analysis were performed as described earlier [2]. Primer oligonucleotides SOD-L1 (AACTGATACGTTTCTTC), SOD-L2 (TACAATAAGGCTATCTG), SOD-L3 (CTAGCGATGAGGTAAA), SOD-L4 (CTCAATTGCTATGGAAGG), SOD-R1 (CTCTTGTTGATTGTCG), SOD-R2 (TAGATAATCCACTGATCC), SOD-R3 (GTATAAATTGACGGG) were deduced from the H. pylori strain 26695 sodB gene [15] (Fig. 2). DNA sequences of the sodB regions in H. pylori strains 151, G27, NCTC11638, P1, 2012, and 022A have been assigned EMBL database accession numbers AJ132687, AJ132688, AJ132689, AJ132690, AJ236919, and AJ251084, respectively. The DNA sequences of the sodB genes in strains 26695, J99, and 60190 were obtained from the EMBL database (accession numbers AE000555, AE001528, and X72618, respectively).

Computational analysis of DNA and protein sequences was performed as previously described [2]. Calculation of the pH was done with the pI/MW tool [17] in the ExPASy Molecular tools collection provided via internet (http://www.expasy.ch/).

2.4. Analysis of FeSOD by SDS-PAGE and immunoblot

Analysis of FeSOD under denaturing conditions was performed by SDS-PAGE and immunoblot as described earlier [13]. The H. pylori FeSOD was detected by immunological cross reaction with polyclonal antiserum raised against FeSOD from E. coli in rabbit [13].

3. Results

3.1. Detection of FeSOD isoforms in H. pylori reference strains and clinical isolates

Analysis by native PAGE and activity staining revealed that the H. pylori reference strains G27 (isoform A), NCTC11638 (A), ATCC43504 (A), 2012 (B) produce electromorphs of FeSOD which were designated isoforms A and B, characterized by a slower and a faster migration behavior, respectively (Fig. 1, I). Strain P1 produces a subtype of isoform B with a migration behavior similar to but slightly slower than isoform B (not shown).

To investigate if further FeSOD isoforms exist within the H. pylori population and to study possible associations with virulence factors and the outcome of disease, 23 clinical isolates originating from a patient group with PUD (16 isolates, vacA s1a/m1, cagA+) and from a control group with NUD (7 isolates, vacA s2/m2, cagA−) were analyzed for FeSOD isoforms by activity staining. Most clinical isolates possessed the FeSOD isoforms A and B (47.8 and 43.5%, respectively). Two of the clinical isolates, 022A and 091C (8.7%), produced another isoform designated C, which migrated faster than isoforms A and B (not shown).

All strains produced a single FeSOD protein (Fig. 1, I) and after repeated analysis, the intensity of activity-stained bands showed no significant strain-dependent variations, indicating that each isolate produced the enzyme in comparable amounts. The Rm values calculated for isoforms...
A (Rm 0.44), B (Rm 0.57), and C (Rm 0.66) differed clearly from those of E. coli MnSOD (Rm 0.18) and Fe-SOD (Rm 0.86).

Comparison of the FeSOD isoforms with the presence or absence of cagA, allele types of vacA, and development of PUD, revealed that no correlations exist. The isoforms were nearly equally distributed in both the control group and the study group. Two isolates with FeSOD variant C were isolated solely from patients with PUD but due to their low frequency this might be a non-significant association.

After repeated subculture the electrophoretic migration behavior of FeSOD remained constant for a given strain and the FeSOD isoform type was not influenced by growth on blood-free BBF agar (HHP or BBF agar, data not shown).

3.2. Analysis of FeSOD isoforms under denaturing conditions

Analysis of FeSOD isoforms A, B and C by SDS-PAGE and by immunoblot with an antiserum raised against E. coli FeSOD [13] demonstrated that under denaturing conditions the isoforms show an identical migration behavior, providing evidence that variations in electrophoretic migration behavior observed under native conditions are not caused by differences in molecular mass (Fig. 1, II).

3.3. Genetic analysis of the sodB gene in strains with different FeSOD isoforms

Sequence analysis of the coding sodB genes from strains G27, NCTC11638, ATCC43504, P1, 151, 2012 and from the clinical isolate 022A, producing the FeSOD isoform types A, B, and C, respectively, revealed that transcriptional organization and length of the reading frame are identical (Fig. 2). The DNA sequence of the coding gene is conserved. Amino acid substitutions caused by strain-specific nucleotide transitions do not concern residues proposed to be involved in catalytic function of FeSOD. Most substitutions are randomly distributed and only one amino acid substitution matches the isoform type of the protein: in all A isoforms aspartate at position 54 is changed to alanine (Fig. 3).

Electrophoretic migration of proteins under native conditions is influenced by the presence or absence of charged amino acids carrying carboxyl or amino groups which in a neutral environment mediate a more negative and or more positive charge, respectively (Fig. 3). Comparison of the protein sequences from different FeSOD isoforms showed that isoform C carries two additional negatively charged residues obtained by the change of glycine and alanine at positions 44 and 94 to aspartate. In contrast, all isoform A proteins have lost a negative charge by a change of aspartate at position 54 to glycine. The FeSOD of strain P1, which migrated slightly more slowly than the other type B isoform proteins, has gained a positive charge by a change of tyrosine at position 71 to asparagine. Other amino acid substitutions have no influence on charge (Fig. 3). These results provide evidence that the faster electrophoretic migration of isoforms B and C is caused by a more negative total charge of the proteins. This was further confirmed by calculation of pI. Faster migrating FeSODs from strains 022A (pI 5.9), 151 (pI 6.04), 2012 (pI 6.04) have a more acidic theoretical pI than the slower migrating FeSODs from strains NCTC11638, ATCC43504 (both pI 6.19) and G27 (pI 6.35), which is in the neutral pH of the electrophoresis buffer indicative of a faster migration to the anode. From the type B isoforms only the strain P1 protein did not show these characteristic differences in pI (6.19).

The FeSODs from strains 26695 [15] (pI 5.77), J99 [1] (pI 6.04), and 60190 [9] (pI 6.04) for which molecular data on FeSOD are available also show differences in pI. Only FeSOD from strain 60190 was experimentally investigated [9]. The amino acid substitutions, calculated pI, and electrophoretic migration behavior of this FeSOD are similar to isoform B proteins analyzed in this study (Fig. 3).

3.4. Influence of iron and oxidative stress on migration of FeSOD isoforms

To investigate if migration behavior of FeSOD isoforms is influenced by environmental conditions like cofactor

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**Fig. 2.** Schematic representation of the sodB gene locus in *H. pylori* strain 26695. The DNA sequence represented by the upper line corresponds to nucleotide positions 399,970–397,970 in the complete genome sequence of strain 26695. The genes are marked by arrows. The genetic arrangement of the sodB DNA region was found to be identical in strains 26695, NCTC11638, ATCC43504, 2012, G27, P1, and 151. Binding sites for oligonucleotide primers used for PCR and sequence analysis are indicated by black arrowheads.
Concentration or oxidative stress, known to regulate SOD production in other bacteria, strains NCTC11638, ATCC43504, P1, G27, 151, and 2012 were grown on BBF agar supplemented with ferric chloride, desferal, or paraquat, and the FeSOD was analyzed by activity staining. In all strains investigated the conditions applied did not reproducibly change intensity or migration behavior of activity-stained bands (shown for strain 2012 in Fig. 4), indicating that the different migration behavior of the FeSOD isoforms is stable for a given strain as further supported by immunoblot analysis of denatured FeSOD protein analyzed in strain 2012 under the same conditions (not shown).

### 4. Discussion

The observation that all *H. pylori* isolates produced a single FeSOD is in good agreement with findings of earlier studies [9,13], and with the fact that a single sodB gene homolog is present in the genomes of strains 26695 and J99 [1,15]. Differences in electrophoretic migration behav-
The survival of successful, indicating that FeSOD might be essential for formation of FeSOD isoforms in SOD subunit [8,10]. A similar mechanism cannot be excluded for formation of FeSOD isoforms in C. jejuni and H. pylori strain 2012 grown on BBF agar without modification (lane 2) or supplemented with ferroc or ferrous iron (1 mM each, lanes 3 and 4), 15 μM desferal (DF, lane 5), or paraquat (PQ, lanes 7 and 8) at concentrations of 1 and 0.5 μM, respectively. FeSOD is marked by the arrow.

Variations in electrophoretic mobility have been reported in several bacterial species [3,4,6]. Environmental stimuli and subcultivation did not influence migration characteristics of H. pylori even under normal growth conditions as shown earlier for SOD of Legionella pneumophila [12]. In other bacterial species SOD production is regulated by cofactor concentration and/or by oxidative stress [3,4,6]. Environmental stimuli and subcultivation did not influence migration characteristics of H. pylori FeSOD isoforms, indicating that the FeSOD polymorphisms identified here represent stable and strain-specific markers which extend our repertoire to differentiate among strains.

However, equal distribution of FeSOD isoforms in control and study groups and the lack of reproducible strain-specific differences in the intensity of activity-stained bands provided no evidence for associations of individual isoforms with virulence determinants or with the outcome of disease.

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