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Diversity of Superoxide-Dismutases Among Clinical and Soil Isolates of *Streptomyces* Species

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Abstract. Comparison of the nature, activity, and cellular localization of superoxide-dismutases (SOD) from soil and clinical isolates of *Streptomyces* species was investigated to identify possible factors that could account for the pathological role of the strains isolated from human lesions. Results showed that all of the studied strains possessed a cytoplasmic Ni-SOD. This particular SOD, found in isolates from patients, could be a new taxonomic criterion to identify *Streptomyces* species with greater precision. A second minor SOD, assimilated to an Fe/Zn-SOD, was detected in some strains, but no relationship was established between the presence of this enzyme and the clinical origin of the strains.

Superoxide dismutases (SODs) that catalyze the conversion of the superoxide anion into hydrogen peroxide and oxygen are the key enzymes in the defense of bacterial cells against reactive oxygen species. Most bacteria contain two types of cytosolic SODs according to the nature of the metal cofactors: iron (Fe-SOD) and manganese (Mn-SOD). These Fe-SOD and Mn-SOD share high similarities, but in *Escherichia coli* the Fe-SOD is expressed constitutively, while Mn-SOD is induced by aerobiosis and iron limitation [14]. Some pathogenic and non-pathogenic, Gram-negative bacteria produce an unusual periplasmic SOD containing copper and zinc that is evolutionarily distinct from Fe- and Mn-SOD [13]. The peripheral localization of this Cu/Zn-SOD plays a major role in defense against extracellular superoxide from the environment or the infected host [6, 11]. Several reports also emphasize the importance of these SODs as possible determinants of pathogenesis in Gram-positive bacteria such as *Listeria monocytogenes* [15], *Nocardia asteroides* [1], and *Mycobacterium tuberculosis* [18]. For the latter two, a role in pathogenicity has been shown for surface-associated SOD and its extracellular production.

Recently, a novel type of SOD containing nickel as cofactor has been found in the filamentous Gram-positive bacterium *Streptomyces* spp. [4, 16, 17]. This Ni-SOD is distinct from the above three groups of SODs on the basis of amino acid sequence. *Streptomyces coelicolor* produces an additional SOD containing iron and zinc. N-terminal amino-acids and enzymatic properties suggest that this Fe/Zn-SOD is similar to either Fe-SOD or Mn-SOD [5].

The main purpose of this study was to investigate and compare the SOD characteristics of soil isolates of *Streptomyces* spp. in our laboratory collection and clinical *Streptomyces* spp. strains isolated from patients. The main objective was to study whether these enzymes could be indicators of the pathogenicity of these bacteria.

Materials and Methods

Strains and growth conditions. The bacterial strains used in this study are listed in Table 1. The strains designated CNR (National Reference Center for Human Mycosis, Antifungal Agents and Actinomycetes, Pasteur Institute, Paris, France) were isolated from patients with various pulmonary or systemic lesions from which the *Streptomyces* species were strongly suspected to be etiologic agents. All strains were identified to the genus level by detection of LL-diaminopimelic acid in their cell walls and using classical taxonomic methods.

In general, the *Streptomyces* spp. were grown in 500-ml serum flasks containing 100 ml of a complex medium (per liter: yeast extract, 4 g; malt extract, 10 g; glucose, 4 g). For iron and nickel depleted

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Table 1. Origin of the *Streptomyces* strains, SOD activity from cytosolic extracts, and mobility of the enzymes on 10% native PAGE

Strain	Origin or clinical feature	U/mg of proteins			Mobility (R_f)
		+Fe + Ni	−Fer	−Ni	
Streptomyces sp.CNR 92.0578	Blood culture	13.5	12.0	1.0	0.33
S. coelicolor A(3)2	soil	15.5	ND	ND	0.36–0.54
S. lividans TK 23	soil	12.0	15.0	1.4	0.36–0.54
Streptomyces sp.CNR 95.0903	Auricular abscess	11.5	ND	ND	0.36
Streptomyces sp.CNR 95.1694	Blood culture	12.4	15.0	4.1	0.36–0.52
S. ambofaciens DSM 40697	soil	ND[a]	ND	ND	0.44
S. aureofaciens ATCC 10762	soil	ND	ND	ND	0.44
S. chromofuscus DSM 40273	soil	ND	ND	ND	0.44
S. griseoflavus ETH 9578	soil	8.1	7.9	3.7	0.44
S. viridosporus ATCC 39115	soil	9.5	9.5	1.2	0.44
S. viridosporus DSM 40243	soil	8.2	ND	ND	0.44–0.52
Streptomyces sp.CNR 93.1381	Blood culture	9.0	10.2	4.3	0.44
Streptomyces sp.CNR 94.1066	Blood culture	19.0	ND	ND	0.44–0.59
Streptomyces sp.CNR 95.1133	Bone abscess	9.0	ND	ND	0.44–0.61
S. scabies DSM 40078	Phytopathogen	17.1	17.8	2.1	0.52
S. somaliensis DSM 40267	Mycetoma	15.3	18.9	2.3	0.52
Streptomyces sp.CNR 92.0665	Broncho-alveolar fluid	8.5	ND	ND	0.52
Streptomyces sp.CNR 94.0075	Cutaneous abscess	14.0	19.8	2.0	0.52
Streptomyces sp.CNR 95.0510	Transtracheal aspiration	13.2	ND	ND	0.52
Streptomyces sp.CNR 95.1458	Cerebrospinal fluid	17.3	ND	ND	0.52

[a] ND = Not determined.
The cells were cultured in a defined medium containing 36 μM $FeCl_3$ and/or 40 μM $NiCl_2$, for 2 days when all elements were present or 3 days when one element was lacking. The assays were performed in duplicate, and their mean values are presented.

conditions, the glassware was treated with 10% nitric acid, and the cells were cultured in a chemically defined medium (per liter: K_2SO_4, 4 g; K_2HPO_4, 3 g; NaCl, 1 g; NH_4Cl, 5 g). The solution was passed through a Chelex-100 resin (Bio-Rad), adjusted to pH 7.2, and the essential ions—320 μM $MgSO_4$, 7 H_2O; 70 μM $ZnSO_4$, 7 H_2O; 0.2 μM $CuSO_4$, 5 H_2O; and 0.2 μM $MnSO_4$, 2 H_2O—were added. Before use, $CaCl_2$ (0.75 mM), glucose (0.25%), and yeast extract (0.005%) were added to the medium. When needed, 36 μM $FeCl_3$ and 40 μM $NiCl_2$ were added. Inoculations were made with 2- to 4-week-old spores harvested from cultures grown on the solid complex medium, blended with glass beads (diameter 2 mm), and filtered through glass wool. About 10^6 spores were inoculated per flask. After 24 h of static incubation at 30°C, the cultures were transferred on a rotary shaker (100 rpm) and allowed to grow for 2–3 days.

Preparation of bacterial extracts. The bacterial pellets were collected on nylon filters, washed with 50 mM sodium phosphate buffer pH 7.8, suspended in 5 ml of the same buffer, and stored at −20°C. Frozen cells were thawed and disrupted by sonication for 9 min with intermittent 3-s pulses (sonifier: Sonicator XL2020). After 10 min of centrifugation at 5000 *g*, the supernatant (crude extract) was mixed with 2 mM phenylmethylsulfonyl fluoride and subjected to ultracentrifugation (30 min at 30,000 *g*, then 1 h at 105,000 *g*). The membrane-containing fraction was suspended in 1 ml of 50 mM sodium phosphate buffer pH 7.8.

Biochemical assays. Iron depletion was controlled with the presence of siderophores in the growth medium [12]. Protein concentration was determined by the Lowry's method using bovine serum albumin as a standard [7]. SOD activity was estimated by the xanthine/xanthine-oxidase procedure with 0.05 mM nitro blue tetrazolium (NBT), 0.1 mM xanthine, 0.008 $U \cdot ml^{-1}$ xanthine-oxidase (Sigma, St. Louis, MO, USA) [2]. The samples were incubated for 1 h at 30°C before reading the reduction of NBT at 560 nm. The amount of SOD required to inhibit the reduction rate of NBT by 50% was defined as one unit of activity. Results were expressed as units/mg of total proteins.

Polyacrylamide gel electrophoresis (PAGE) and staining for SOD activity. Cell extracts were separated on a 10% non-denaturing polyacrylamide gel by using a buffer system without sodium dodecyl sulfate (SDS) in the gel or in the running buffer. Staining for SOD activity was monitored as described by Beauchamp and Fridovich [2].

Results and Discussion

The SOD activities obtained from crude extracts of the bacterial strains grown in the defined medium supplemented with 36 μM $FeCl_3$ and 40 μM $NiCl_2$ are given in Table 1. Yeast extract incorporated in low concentration in the medium was only intended to enhance spore germination and to give homogeneous growth rates. Cells were harvested after 2 or 3 days of shaking because of the reduction of SOD activity in older cultures. Pellet development in shaken cultures can account for these results as in large pellets; the inner and external cells probably exhibit different enzymatic activities.

SOD activity, measured in cytoplasmic extracts of cells grown in non-depleted medium, was between 8.1 and 19 units/mg of protein, and the extracts from the clinical *Streptomyces* spp. did not exhibit significantly more activity than the soil strains. No activity was

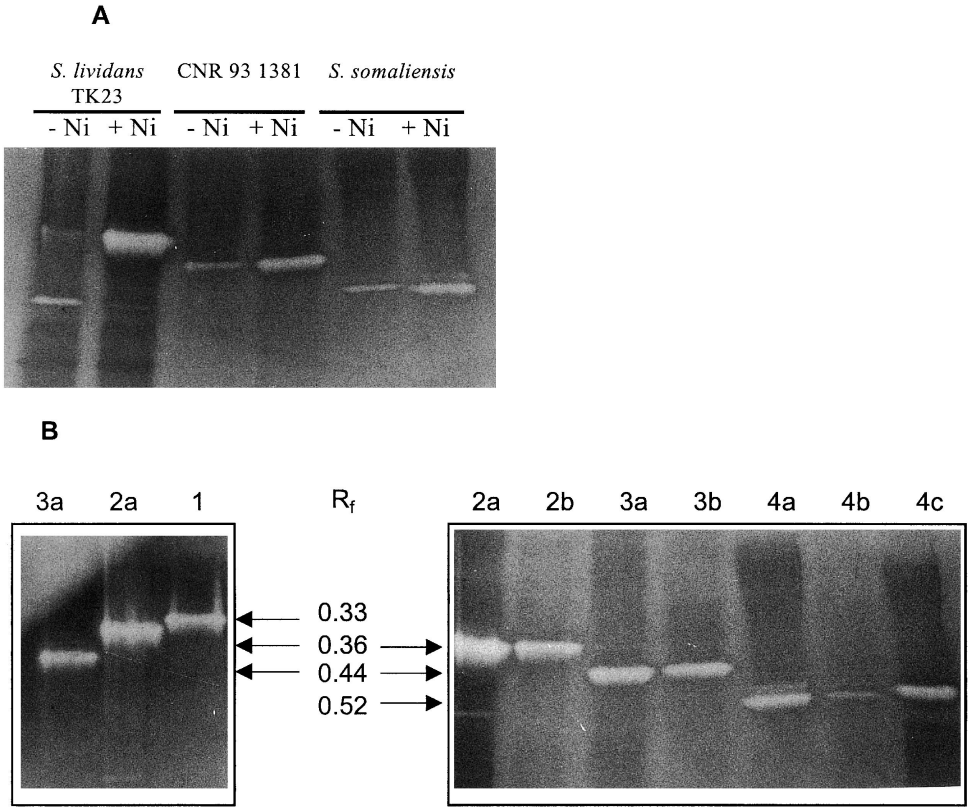

Fig. 1. SOD activity by *Streptomyces* spp. on 10% native PAGE; 20 µg of protein was loaded per lane. A: cytoplasmic extracts of cells were cultured with or without nickel. B: cells were cultured in a chemically defined medium containing 40 µM $NiCl_2$. 1, *Streptomyces* sp. CNR 92 0578; 2a, *Streptomyces lividans* TK23; 2b, *Streptomyces* sp. CNR 95 1694; 3a, *Streptomyces viridosporus* ATCC 39 115; 3b, *Streptomyces* sp. CNR 95 1133; 4a, *Streptomyces somaliensis*; 4b, *Streptomyces* sp. CNR 94 0075; 4c, *Streptomyces scabies*.

detected in the culture medium or in the cell-wall fraction. Consequently, the SOD activity of all the *Streptomyces* spp. strains was only cytoplasmic.

Generally, when the cells were grown in the complex medium, the total activity represented less than 15% of that obtained by using the minimal medium supplemented with iron and nickel, and the same cytoplasmic localization of SOD was detected.

Without iron in the growth medium, depletion was verified by siderophore production because these small-molecular-weight molecules are produced and secreted in response to iron starvation; the presence of available iron acts as a co-repressor for the regulation of biosynthesis of siderophores. In these conditions, the specific activity was roughly the same as with iron complementation; meanwhile, a decrease in cell biomass was noted. Without nickel, SOD activity was very low for all the tested strains, and the decrease represented up to 90%.

For *S. viridosporus* DSM 40243, *S. lividans*, *S. coelicolor*, *Streptomyces* sp. CNR 94.1066, CNR 95.1133, and CNR 95.1694, two SODs were separated on PAGE. Only one enzyme was detected for the other *Streptomyces* species (Table 1, Fig. 1).

The experiments monitored for Ni-depletion showed that, contrary to the minor SOD, the major SOD produced by all strains was Ni-dependent. Dependent upon the strains, four distinct patterns of migration for the main SOD were observed on PAGE (Fig. 1). The strains were thus classified into four groups with their respective R_f being 0.33, 0.36, 0.44, and 0.52. Under the same conditions, the Mn-SOD of *E. coli* moved at $R_f = 0.24$ and Fe-SOD at $R_f = 0.42$. These four bacterial groups comprised clinical or soil strains (Table 1). The group with the SOD $R_f = 0.52$ included the human pathogenic strain *S. somaliensis*, the plant pathogenic strain *S. scabies*, and four clinical isolates identified as CNR 92 0665, CNR 94 0075, CNR 95 0510, and CNR 95 1458.

The SOD present in all of the *Streptomyces* spp. tested was dependent on the presence of nickel in the growth medium and corresponded to the Ni-SOD described by Youn et al. [17], whereas the second SOD, found in some strains, was probably the Fe/Zn-SOD as reported by Kim et al. [4] and Youn et al. [17].

The presence of Ni-SOD in clinical strains confirmed the identification of the bacteria isolated from humans as members of the genus *Streptomyces*. This common

property of *Streptomyces* spp. to synthesize a Ni-SOD seems to be characteristic of members of this genus. In contrast, phylogenetically closely related bacteria produce a classical SOD: Mn-containing enzyme, such as *Nocardia* [1]; or two types of SOD such as a Mn-SOD for most species of *Mycobacteria* [8–10] and a Fe-SOD for *M. tuberculosis* [3]. The presence of a Ni-SOD could be a useful additional taxonomic criterion during early diagnosis for the difficult distinction between *Streptomyces* and *Nocardia* species.

However, no conclusion on bacterial pathogenicity can be drawn regarding the localization of SOD. According to the native PAGE migration patterns of Ni-SOD, a similar R_f is observed for clinical and soil strains which can belong to a single group.

In contrast to the Ni-SOD, the minor Fe/Zn-SOD seems to be accessory and species relationless. The two closely related species *S. lividans* and *S. coelicolor* produced both enzymes, whereas the two *S. viridosporus* strains don't give similar results.

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