Abstract  The latency phase, growth rate, cell yield and end-products of Lactobacillus sanfranciscensis CB1 were affected by oxygen and the supply of 225 μM Mn²⁺. Mn²⁺ was especially related to the highest substrate consumption. Aerobiosis and Mn²⁺ supply were responsible for the highest superoxide dismutase activity. An auto-inhibitory accumulation of H₂O₂ meant that the survival of air-grown cells supplied with Mn²⁺ rapidly decreased during the stationary phase. As shown by sodium dodecyl sulfate/polyacrylamide gel electrophoresis, Mn²⁺ supply influenced protein expression. As shown by non-denaturating zymograms, Lb. sanfranciscensis CB1 expressed an approximately 12.5-kDa superoxide dismutase, which is probably Mn-dependent. The enzyme was insensitive to H₂O₂ treatment, was not induced by the presence of paraquat under aerobic conditions and was relatively stable at pH 4.0. Sourdoughs that contained high levels of oxygen enhanced cell growth, acidification and acetic acid production by Lb. sanfranciscensis CB1.

Introduction
Although considered anaerobic, micro-aerophilic or aero-tolerant, lactic acid bacteria may take advantages or change their metabolism in response to oxygen (Teuber 1993). Reduced pyridine nucleotide oxidases and pyruvate oxidase are common in lactic acid bacteria (Condon 1987). With the stepwise reduction of O₂ to H₂O, oxidase enzymes generate toxic intermediates such as superoxide anions (O₂⁻) and H₂O₂.

Lactic acid bacteria can deal with O₂⁻ by either a superoxide dismutase (Sod) or a high internal Mn²⁺ concentration (Archibald and Fridovich 1981). In prokaryotes, three types of Sod are distinguished by their metal cofactors: Cu-Zn, Fe, or Mn. Lactococci and streptococci contain true Mn-containing Sod and none of them uses a non-proteinaceous manganese compound to scavenge O₂⁻ (Chang and Hassan 1997; Sanders et al. 1995). Pseudo-Mn-containing catalase has only been purified in Lactobacillus plantarum (Igarashi et al. 1996) and heme catalase is moderately diFuse in strains belonging to virtually all genera of lactic acid bacteria (Engesser and Hammes 1994). Most of the lactic acid bacteria use NADH peroxidase activity to decompose H₂O₂ (Condon 1987). NADH peroxidase potentially fulfills the role of heme-containing catalases by reducing intracellular levels of H₂O₂ while also regenerating oxidized pyridine nucleotide (Ross and Claiborne 1997).

Except for Lb. plantarum (Götz et al. 1980), the responses of lactic acid bacteria to oxygen have not been studied in lactobacilli. Lactobacillus sanfranciscensis is a key sourdough lactic acid bacterium (Gobbetti and Corsetti 1997). Although it has been shown that its growth and metabolism are affected by the availability of electron acceptors such as fructose and oxygen (Gobbetti et al. 1995; Stolz et al. 1995a), the response of Lb. sanfranciscensis to oxygen needs to be explained and results applied during sourdough fermentation.

This paper deals with Lb. sanfranciscensis CB1 and shows (i) the response to oxygen and the synthesis of a true Sod enzyme; (ii) the effect of Mn²⁺ supply on growth and metabolism, and (iii) the application of some results in sourdough fermentation.

Materials and methods
Microrganism and culture conditions
Lactobacillus sanfranciscensis CB1, from the culture collection of the Institute of Dairy Microbiology, Agriculture Faculty of Peru-
gia, Italy, was cultivated twice on sourdough bacteria medium (SDB): 20 g/l maltose, 6.0 g/l tryptone, 3.0 g/l yeast extract, 0.3 g/l Tween 80, final pH 5.6 (Kline and Sugihara 1971) at 30°C for 24 h. Cells were then inoculated (4%) in modified SDB, which contained 10 g/l maltose, 2.6 g/l K2HPO4, 4 g/l KH2PO4; the final pH was 6.2. Cells kept constant at pH 6.2 were made by the on-line addition of 1.0 M NaOH. When added, Mn2+ (as MnSO4·4H2O) was optimal at 225 μM. Anaerobic cultures were made in screw-cap bottles with a little, closed hole by which N2 was insufflated every 2 h of incubation (at a pressure of 0.4 bar). Aerated cultures were made in 300-ml shaking flasks in an orbital shaker (150 rpm) and air was insufflated every 2 h of incubation (at a pressure of 0.4 bar). Cultures were incubated at 30°C for 24–48 h.

Preparation of cell extracts, PAGE and Sod activity detection by zymogram

Cells of Lb. sanfranciscensis CB1 were harvested by centrifugation (5500 g for 5 min at 4°C) in the exponential phase of growth (8–16 h depending on the culture conditions). The cell extract was produced by lysozyme and sonication treatments (Gobbetti et al. 1995), dialyzed against distilled water for 24 h at 4°C and freeze-dried. The cell extract was used for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the protocol of Laemmli (1970). Each sample loaded contained 25 μg protein. Nondenaturing PAGE was conducted similarly, except that SDS and mercaptoethanol were omitted. Prior to loading, samples were incubated at 37°C for 15 min. Polyacrylamide gels were stained by Coomassie brilliant blue (Sanders et al. 1993). Sod activity in nondenaturing gels was determined according to the protocol of Beauchamp and Fridovich (1971), which used riboflavin as a reducing agent. The molecular mass of the Sod enzyme in the nondenaturing system was determined indirectly by electrophoresing molecular mass markers (range 14.0–454.0 kDa) and unknown proteins on a set of gels of various polyacrylamide concentrations (Sigma Chemical Co. St. Louis, Mo.).

Sod activity and H2O2 determination

Sod activity was determined by measuring the inhibition of superoxide-anion-induced reduction of nitroblue tetrazolium (NBT) according to Gerlach et al. (1998). Cell extracts were used at the protein concentration of 200 μg/ml. The activity was calculated as the percentage inhibition of NBT reduction in the presence of cell-free extracts. One unit (U) of activity corresponded to 1% inhibition/min reaction.

Sod activity of cells grown in aerobic, with shaking (150 rpm) and with 225 μM Mn2+ supply, was further characterized. The effect of H2O2 on Sod activity was assayed by treatment (1 h) of the cell-free extract with various concentrations (1, 10 and 30 mM) of H2O2. The H2O2-treated extracts were dialyzed against 0.05 M phosphate buffer, pH 7.0, for 12 h, before the assay for Sod (Chang and Hassan 1997).

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The pH stability was examined after pre-incubation of the cell-free extract at pH 4.0 (0.1 M, sodium acetate buffer) for 12 h at 4°C. After dialysis against 0.05 M phosphate buffer, pH 7.0, for 12 h, the Sod activity was assayed. To determine the heat stability, portions (100 μl) of the cell-free extract were heated in sealed capillary glass tubes at different temperatures (50–65°C) for different periods of time.

Aerated cells supplied with Mn2+ were grown in the presence of 0.1 mM paraquat to determine the effect of redox cycling compounds capable of generating O2̅ (Chang and Hassan 1997). Cell extracts and Sod activity were assayed as reported above. H2O2 was determined by the method of Kingsley and Getchell (1960) by measuring the absorbance of the oxidized o-diaminodisidine at 410 nm. Cultures were centrifuged at 5500 g for 5 min and the supernatant was used for analysis. The H2O2 concentration was calculated by a standard curve determined on SDB broth containing various amounts of H2O2.

Sourdough fermentation

The characteristics of the type 0 wheat flour were as follows: moisture 12.8%, protein (N×5.83) 10.6% dry matter (d.m.), fat 1.8% d.m. and ash 0.6% d.m. The dough contained 200 g wheat flour, 44 ml tap water and 26 ml cellular suspension of Lb. sanfranciscensis CB1 (1·109 cfu/ml). Before mixing, cell suspensions were insufflated for 5 min with air or N2. The doughs had a dough yield of 135. During mixing, by a continuous mixer (60 g, dough mixing time = 10 min), doughs were also insufflated by air or N2. The sourdough fermentation was at 30°C for 24 h.

Determination of cell number, carbohydrates and metabolites

The cell number of Lb. sanfranciscensis CB1 was estimated indirectly by the As2O3 or by plating on SDB/agar medium, after 72 h at 30°C. Maltose, D- and L-lactic acid, acetic acid and ethanol were determined by enzymatic methods (Boehringer-Mannheim, Milan, Italy).

Results

Lb. sanfranciscensis CB1 growth and metabolism

Agitation, aeration and Mn2+ availability reduced the length of the lag phase and increased the growth rate and cell yield of Lb. sanfranciscensis CB1 (Fig. 1a). The viable cell count of an aerobic culture supplied with Mn2+ markedly decreased from 2.2·109 to 1·107 cfu/ml after approaching the stationary phase (12 h), while anaerobic cultures showed no reduction in cell numbers during 48 h.

Agitation and especially the Mn2+ supply were related to the fastest substrate consumption (Fig. 1b). At 12 h the consumption of maltose was 26.2 mM compared to 16.7 mM in aerated cultures with or without the cation. At 12 h, the mM production of ethanol or acetic acid/mM maltose consumed were 1.436 and 0.160 in anaerobiosis compared to 0.935 and 0.679 in aerobiosis. In aerobicosis and with shaking, the supply of Mn2+ gave the same millimolar ratio for C2 (acetic acid and ethanol) plus C3 (lactic acid)/mM maltose consumed as in its absence (3.33 versus 3.28 respectively) but differences in C2 plus C3 production (87.0 mM versus 57.0 mM), because of the variation in the maltose consumption. As a consequence, aerobiosis and the supply of Mn2+ produced a noticeable concentration of acetic acid (17.66 mM versus 13.91 mM) in the presence of lactic acid (45.11 mM versus 28.77 mM) and ethanol (24.23 mM versus 14.32 mM) that was still higher than those produced without cation addition.

Denaturing and nondenaturing PAGE and Sod activity and characterization

Cell extracts of Lb. sanfranciscensis CB1 were analyzed by SDS-PAGE (Fig. 2). The level of protein expression differed with the supply of 225 μM Mn2+ (lanes 1, 3 and 5 compared to 2, 4 and 6). All these cell extracts were...