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Molecular Clues To Understand the Aerotolerance Phenotype of *Bifidobacterium animalis* subsp. *lactis*

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Oxygen is one of the abiotic factors negatively affecting the survival of *Bifidobacterium* strains used as probiotics, mainly due to the induction of lethal oxidative damage. Aerobic conditions are present during the process of manufacture and storage of functional foods, and aerotolerance is a desired trait for bifidobacteria intended for use in industry. In the present study, the molecular response of *Bifidobacterium animalis* subsp. *lactis* IPLA4549 to aerobic conditions is presented. Molecular targets affected by oxygen were studied using two-dimensional electrophoresis (2DE) and quantitative reverse transcriptase (qRT) PCR. Globally, oxygen stress induced a shift in the glycolytic pathway toward the production of acetic acid with a concomitant increase in ATP formation. Several changes in the expression of genes coding for enzymes involved in redox reactions were detected, although the redox ratio remained unaltered. Interestingly, cells grown under aerobic conditions were characterized by higher activity of coproporphyrinogen III oxidase, which can directly detoxify molecular oxygen, and by higher NADH oxidase specific activity, which can oxidize NADH using hydrogen peroxide. In turn, this is in agreement with the glycolytic shift toward acetate production, in that more NADH molecules may be available due to the lower level of lactic acid formation. These findings further our ability to elucidate the mechanisms by which *B. animalis* copes with an oxygen-containing atmosphere.

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (3). Many probiotic strains present little resistance to most of the technological processes used by the food industry, limiting their use in most food product categories, with the exception of dairy products (29). However, it is known that certain strains can adapt to industrial stresses (9). It is noteworthy that aerobic conditions are present during the whole process of manufacture and storage of functional foods. Oxygen tolerance is thus a desired trait for strains used in industry, since it may guarantee high bacterial survival in end products.

Several strains of bifidobacteria are widely used as probiotics. Usually, aerotolerance displays a high species-to-species variation in *Bifidobacterium*, and several species show an aerotolerant phenotype if a reducing agent, such as L-cysteine, is included in the liquid medium (5, 23). In this regard, *Bifidobacterium animalis* subsp. *lactis* is one of the most aerotolerant species (37). This fact, together with its good resistance to the harsh human gastrointestinal tract conditions, makes the species one of the most widely used bifidobacteria in the functional food industry (14, 22). Although described as strictly anaerobic, this has to be taken in the sense of their inability to form colonies on agar plates exposed to air (42). In fact, there is growing evidence that some species, such as *B. animalis*, are aerotolerant (5, 23, 42) or even able to form colonies on the surface of solid agar medium in the presence of air, such as *Bifidobacterium psychraerophilum* (38). In this sense, bifidobacteria were classified more than 40 years ago into three groups according to their aerotolerance (8).

Loss of viability due to oxygen toxicity is considered one of the main abiotic factors affecting the survival of *Bifidobacterium* strains, mainly due to the induction of lethal oxidative damage in the microorganisms (21). Aerobic conditions are present during the process of manufacture and storage of functional foods. Tolerance for oxygen is of paramount importance for a microorganism intended to be used in functional food products, as in the case of probiotic bacteria, since this trait may guarantee both its stability and viability in end products (19).

The deleterious effects of oxygen on bacterial physiology are essentially due to oxidative damage induced by the formation of the so-called reactive oxygen species (ROS). Bacteria have developed several mechanisms for counteracting these oxidative effects, among which glutathione production and antioxidative enzymes, such as catalase, superoxide dismutase, peroxidases, and oxidases, are worth mentioning (13, 24, 25).

Members of the phyla *Actinobacteria* and *Firmicutes* can counteract the toxic effects of ROS with the production of flavoproteins, which in turn results in the production of compounds with high antibacterial activity, such as hydrogen peroxide (19). It is known that certain lactic acid bacteria can decrease ROS accumulation in foods, and for this reason, they are considered antioxidant microorganisms (18). In bifidobacteria, aerotolerance seems to be dependent on the presence of certain NADH oxidases and peroxidases capable of detoxifying the cells (35, 36).

In the present work, the aerotolerance of the strain *B. animalis* subsp. *lactis* IPLA4549 has been studied using a complementary genomic, proteomic, and physiological approach. Two-dimensional electrophoresis (2DE) and quantitative reverse transcriptase (qRT) PCR were used for identifying changes in target genes and proteins as affected by aerobic conditions during growth. Physiological parameters, such as the redox ratio, intra-

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TABLE 1 Sequences of primers for qRT-PCR used in this study

Spot/protein[a]	Primer name	Sequence (5′→3′)	Primer T_m[b] (°C)
101	101-RT-uni	CTTCAACGCTCAGGAGATGA	63.14
	101-RT-rev	GTCCTTCTTGACGATCGGA	62.85
163	163-RT-uni	GTCGTAGTACGCGTAGGTG	58.36
	163-RT-rev	GAACCGCGACGACGAG	64.61
169	169-RT-uni	GATCGATCATGTTCTGAGCAC	62.27
	169-RT-rev	GAGGAGATCCTCCCGAAG	61.33
175	175-RT-uni	GACTGGACATCGGTGAGCA	65.23
	175-RT-rev	GAAGCCCACGACATCATGAAC	66.65
180	180-RT-uni	CAATCATCGGTTACGGCTC	63.02
	180-RT-rev	CTTCCGGAACGCTCTTGAC	64.86
Possible reductase	PR-RT-uni	GTGAAGTGCGTGTTGTTGAG	62.03
	PR-RT-rev	TCCTGCCGATAGCCGCATC	70.66
Thioredoxin peroxidase	ThioperF	CTCCCCCGACGATGTGAGG	70.32
	ThioperR	AGGTCGTTGCTCTTGCGAAA	67.03
Thioredoxin	ThioF	CGCAGATTTCGAACAGGCAG	68.02
	ThioR	AAATCGAACAGCGCGATCTC	66.43
Thioredoxin reductase	ThioredF	CAGATCATGGTCGAGCGCA	68.83
	ThiredR	CATGAGCAGGTTGATCTTCGG	66.82
Coproporphyrinogen III oxidase	CoproIIIF	GAATGGCAGTTGGCACATGG	68.69
	CoproIIIR	GAAAACGGTGGATGCAGGC	67.78
NADH oxidase	NAOX-F	CGCCGGTCTGCAAATCG	69.13
	NAOX-R	TCAGTGACGTCGGTGTGCA	68.31

[a] 101, F_1F_o-ATPase subunit alpha; 163, probable UDP-galactopyranose mutase; 169, transketolase; 175, transaldolase; 180, ketol-acid reductoisomerase.
[b] T_m, melting temperature.

cellular ATP pool, end product concentrations of the glycolytic pathway, and oxygen-dependent coproporphyrinogen III oxidase and NADH oxidase activities, were also estimated in cultures grown under aerobic and anaerobic conditions. Overall, these results allowed us to shed some light on the mechanisms responsible for aerotolerance in *B. animalis* subsp. *lactis*.

MATERIALS AND METHODS

Bacterial growth conditions. The strain *B. animalis* subsp. *lactis* IPLA4549 was cultured in MRSC (MRS [Biokar Diagnostics, Beauvais, France] with 0.05% [wt/vol] L-cysteine [Sigma Chemical Co., St. Louis, MO]) and incubated for 24 h at 37°C in different systems, implying different rates of oxygen diffusion to the growing culture. These systems were an anaerobic chamber MG500 (anaerobic conditions; Don Whitley Scientific, West Yorkshire, United Kingdom) under 10% (vol/vol) H_2, 10% CO_2, and 80% N_2; a conventional heater (slightly aerobic conditions); or an Excella E24 Incubator rotary shaker (aerobic conditions; 200-ml cultures in 500-ml flasks; 250 rpm) (New Brunswick Scientific, Edison, NJ). Under these three conditions, growth was monitored by following the optical density at 600 nm (OD_{600}) of the cultures during the experiment. Cells were always harvested at mid-exponential phase under the three different conditions mentioned above. Unless otherwise specified, at least three biological replicates were performed for all the experiments described.

Protein manipulation and 2DE. Pellets were washed twice in 0.1 M Tris-HCl buffer, pH 7.5, and resuspended in 5 ml of 1 M Tris-HCl buffer, pH 7.5, and lysed through a cell disrupter (Constant Systems Ltd., Daventry, United Kingdom) using a pressure of 2.05×10^8 Pa. Unbroken cells and cell debris were removed by centrifugation (4,500 × *g*; 4°C; 15 min), and membrane vesicles/small cell debris were discarded by ultracentrifugation (50,000 × *g*; 4°C; 20 min). The protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) following the manufacturer's instructions. 2DE was carried out as previously described (27, 28), and at least three gels were analyzed for each biological replicate. Images from the gels were obtained and compared using an ImageScanner (Amersham Biosciences, Buckinghamshire, United Kingdom). Spot detection and volume quantitation and normalization were carried out with ImageMaster 2D Platinum software (version 5.0; Amersham Biosciences and Geneva Bioinformatics S.A.). The volume of each spot was calculated and normalized by referring values to the sum of total spot volumes within each gel. Student's *t* test for unpaired samples was applied, and spots showing statistically significant variation between the two conditions were selected for identification. Proteins were identified at the Proteomics Core Facility of the Centro Nacional de Investigaciones Cardiovasculares (Madrid, Spain), following standard protocols.

Quantitative RT-PCR. The relative expression of the genes whose corresponding proteins showed differences in their production under aeration versus anaerobiosis was determined through qRT-PCR at the mid-exponential phase of growth (OD_{600}, 0.3 to 1.5, depending on the conditions). In addition, six genes coding for other proteins potentially involved in oxygen detoxification were included: thioredoxin peroxidase (GenInfo identifier gi|241196325), thioredoxin (gi|241196792), thioredoxin reductase (gi|241197030), coproporphyrinogen III oxidase (gi|241196329), NADH oxidase (gi|241195709), and a possible reductase (gi|183601851). The protocols for cell lysis, RNA isolation, and cDNA synthesis were performed as previously described (10). qRT-PCR experiments were run in an ABI Prism 7500 Fast-Real-Time PCR system (Applied Biosystems, Foster City, CA). Specific primers were designed for all the proteins (Table 1), and their specificities were verified before the quantitative analysis. At least three independent PCR runs were performed for each cDNA. Relative expression was estimated according to the $\Delta\Delta C_T$ method, using 16S rRNA as an endogenous control employing previously described primers (11, 12). The expression rate was related to the expression of the corresponding genes under anaerobiosis, which was given the value 1.

Organic acid production. Organic acids were determined in buffered cell suspensions. Ten milliliters of IPLA4549, grown until early stationary phase under anaerobic or aerobic conditions, was collected by centrifugation (10,000 × *g* for 15 min) at the same OD_{600} used for the proteomic and qRT-PCR experiments; washed twice with 50 mM Tris-HCl buffer, pH 7.0; and resuspended in 10 ml of 50 mM Tris-HCl buffer, pH 5.6, containing glucose (25 mM). The suspensions were incubated with constant

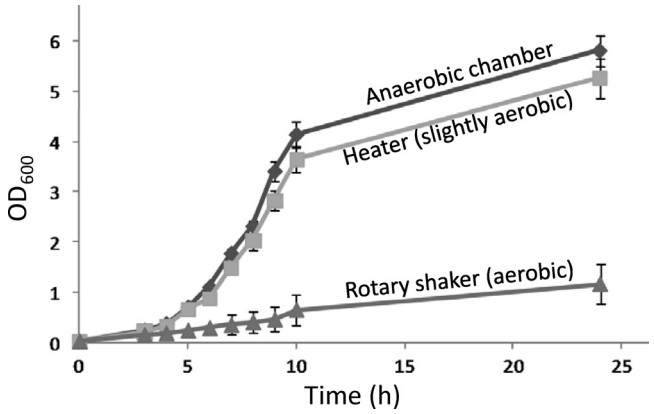

FIG 1 Growth curves of strain IPLA4549 under different experimental conditions, as described in Materials and Methods.

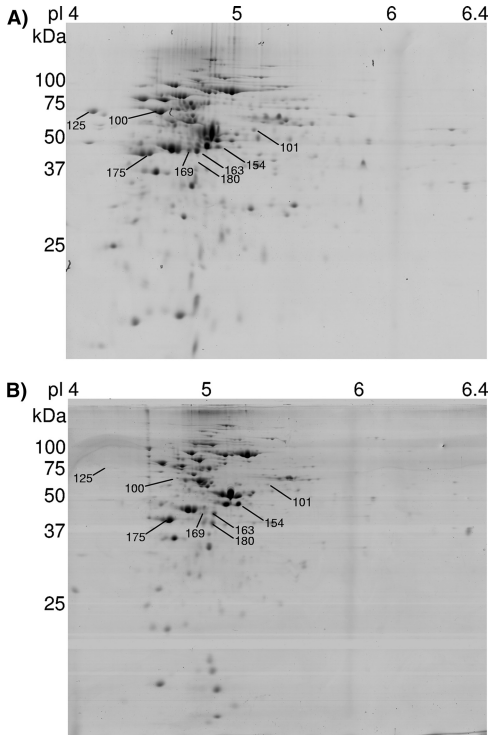

FIG 2 Representative 2DE gels showing the cytoplasmic protein patterns of strain IPLA4549 grown under aerobic (A) or anaerobic (B) conditions. Spots showing statistical differences in their normalized volumes, under aerobic conditions in relation to anaerobic conditions, are marked and numbered.

mild stirring for 4 h at 37°C. The cells were removed from the suspension by centrifugation, and the supernatant was analyzed by high-performance liquid chromatography (HPLC) to determine glucose and organic acid levels.

Determination of the amounts of glucose and acetic and lactic acids in the cell supernatants was carried out essentially as previously described (26, 27, 28). Briefly, a chromatographic system composed of an Alliance 2690 module injector, a Photodiode Array PDA 996 detector, a 410 differential refractometer detector, and Millenium 32 software (Waters Corporation, Milford, CA) was used. Samples (50 μl) were separated under isocratic conditions using an HPX-87H Aminex ion-exchange column (Hewlett Packard, Palo Alto, CA) with diluted sulfuric acid as the mobile phase. Standard solutions of organic acids and glucose were used as controls for both identification and quantification. The results shown are the means of at least three biological replicates per condition and are expressed as concentrations per cell OD_{600} (mM/OD_{600} unit). Finally, the acetic/lactic acid ratios and the carbon balance of the pathway {[2 × (acetic acid produced) mM + 3 × (lactic acid produced) mM]/6 × (glucose consumed) mM} were also calculated.

Estimation of the redox balance by fluorescence spectroscopy. The fluorescence properties of buffered cell suspensions (OD_{600}, 0.6) in 50 mM Tris-HCl buffer, pH 7.0, were monitored in an Eclipse Fluorescence Spectrophotometer (Varian, Inc., Palo Alto, CA). The intensity values corresponding to NADH were calculated from the 413-nm emission at an excitation wavelength (λ_{ex}) of 316 nm, whereas for flavin adenine dinucleotide (FAD), the intensity values were calculated from the 436-nm emission at a λ_{ex} of 380 nm, according to the method of Ammor et al. (2). The redox ratio was deduced from the NADH- and FAD-related signals using the following equation: $redox_{ratio} = FAD_{Intensity}/(FAD_{Intensity} + NADH_{Intensity})$ (28).

ATPase activity. The ATPase activity present in inside-out membrane vesicles (5 μg of membrane protein) was calculated from the release of inorganic phosphate, measured colorimetrically after its reaction with sodium molibdate and malachite green, as previously described (31). Inside-out membranes were obtained from independent batch cultures (50 ml) under the different conditions listed above. Pellets were resuspended in 4 ml of 100 mM potassium phosphate buffer, pH 7.0, supplemented with 10 mM $MgSO_4$ and treated with 10 mg/ml lysozyme and 50 units/ml mutanolysin. The suspensions were incubated at 30°C for 4 h with constant stirring. The cells were broken by passage three times through a cell disrupter (Constant Systems Ltd., Daventry, United Kingdom) at 2.05×10^8 Pa. Unbroken cells and cell debris were removed by centrifugation (13,000 × *g*; 4°C; 20 min); membrane vesicles were collected by centrifugation at 125,000 × *g* for 60 min at 4°C and resuspended in 50 mM potassium phosphate buffer, pH 7.0, plus 10% glycerol. The protein concentration of the membrane vesicle extract was measured using a BCA protein assay kit (Pierce, Rockford, IL) following the manufacturer's instructions. Specific F_1F_o-ATPase activity was calculated by preincubating membranes for 10 min at 37°C and subsequently for 60 min on ice with dicyclohexylcarbodiimide (DCCD) (Sigma; final concentration, 0.2 mM). The activity of membrane samples without any inhibitor was measured and used as a control.

Measurement of intracellular ATP content. The intracellular ATP content was determined by bioluminescence as described previously (31). Briefly, the amount of ATP present in the cell lysates, obtained by sonication, was measured by bioluminescence using the ATP Bioluminescence Assay kit HSII (Roche Applied Science, Mannheim, Germany), following the manufacturer's instructions. ATP standard solutions were used to convert arbitrary bioluminescence units into the molarity of ATP. Bioluminescence was measured in a Cary Eclipse Fluorescence Spectrophotometer (Varian, Palo Alto, CA) with a 1-s integration time. ATP levels were corrected by the protein concentration, which was estimated with a BCA protein assay kit (Pierce, Rockford, IL). The results were expressed as nmol ATP mg total protein^{-1}.

Coproporphyrinogen III oxidase activity. According to the BRENDA database, coproporphyrinogen III oxidase can oxidize one molecule of coproporphyrinogen III to protoporphyrinogen IX, with the formation of two molecules of CO_2 and two molecules of H_2O_2 (http://www.brenda-enzymes.org). Protoporphyrinogen IX can be chemically modified by oxidation with H_2O_2 according to the method of Breckau and colleagues (6), a reaction in which it is transformed into protoporphyrin IX. The protocol was performed as follows. Bifidobacterial extracts (100 μl) were oxidized with 30% (vol/vol) H_2O_2 in a volume of 200 μl. Protoporphyrin was determined by fluorimetric detection as described in "Estimation of the redox balance by fluorescence spectroscopy" with an excitation wavelength of 409 nm and an emission wavelength of 621 nm (with slit widths of 5 and 10 nm for excitation and emission, respectively). The arbitrary fluorescence units (AU) corresponding to protoporphyrin

TABLE 2 Proteins showing significant variations in their production as affected by oxygen

Label	Identity	GI[a] no.	OX/A ratio[b]
101	F_1F_o-ATP synthase subunit alpha	gi|183602786	1.42
100	Glucose-6-phosphate isomerase	gi|183601860	9.35
125	Galactokinase	gi|183602136	5.55
154	Glyceraldehyde 3-phosphate dehydrogenase	gi|183602460	0.12
163	Probable UDP-galactopyranose mutase	gi|183602805	2.67
169	Transketolase	gi|183601629	3.42
175	Transaldolase	gi|183601628	0.18
180	Ketol-acid reductoisomerase	gi|183602275	0.00

[a] GI, GenInfo identifier.
[b] Normalized spot volume in aeration (OX) divided by normalized spot volume in anaerobiosis (A). Underproduction, OX/A ratio < 1.4; overproduction, OX/A ratio > 1.4.

were corrected by the amount of protein contained in the extracts and were used for estimating the coproporphyrinogen III oxidase activities in the different bifidobacterial extracts (AU mg total protein^{-1}).

NADH oxidase activity. The NADH oxidase activity was determined in a buffer containing 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, and 200 μM NADH in a final volume of 200 μl (4). A mixture containing 10 μl of each of the different bifidobacterial extracts was incubated for 10 min at room temperature. The degradation of NADH was followed by a decrease in the absorbance at 340 nm. One enzymatic unit of NADH oxidase activity was defined as the amount able to oxidize 1 μmol of NADH per min. Specific NADH oxidase activity (μmol NADH mg total protein^{-1}) was calculated under all the conditions.

Measurement of dissolved oxygen. Oxygen dissolved in the liquid medium was measured using an HI 9146N oxymeter (Hanna Instruments S.L., Eibar, Spain) at 37°C and 6 m above sea level after the same incubation period used for proteomic and qRT-PCR experiments and following the manufacturer's instructions. The results were expressed as ppm of dissolved oxygen (the range from the minimal to the maximal value). At least three independent replicate measurements were carried out for the three conditions tested (anaerobic, slightly aerobic, and aerobic conditions).

Statistical analysis. When required, unpaired Student's *t* tests were used to evaluate the consistency of the data.

RESULTS

Growth of IPLA4549 in the presence of oxygen. Strain IPLA4549 was incubated using different experimental settings in order to get different degrees of aeration, and the growth was monitored by following the OD_{600} of the cultures. Two aerobic conditions were considered: a conventional static heater and a rotary shaker, with the latter leading to higher aeration of the culture. As can be seen in Fig. 1, there were minor changes in the growth of strain IPLA4549 incubated in the heater with respect to the standard anaerobic conditions, although these differences were not statistically significant ($P > 0.05$). On the other hand, growth progressed slowly when the incubation was performed in a rotary shaker, reflecting the inhibitory effect of aeration. The amounts of dissolved oxygen under the three conditions tested was as follows: 0.38 to 0.39 ppm (anaerobic chamber), 1.30 to 1.45 ppm (conventional heater), and 6.54 to 6.60 ppm (rotary shaker).

Changes in protein production. Cultures of IPLA4549 were grown to mid-exponential phase, and cell-free protein extracts were obtained by cell disruption and subsequently resolved and analyzed by 2DE using standard protocols (Fig. 2). Spots showing changes in their normalized volumes were submitted for identification by peptide mass fingerprinting, using the public genomes available in the NCBI nonredundant database. Remarkably, the electrophoretic patterns of protein extracts from cells grown under anaerobic conditions and in the conventional heater were very similar. Therefore, taking into account these results and the similarity in the growth curves of the strains, the incubations in the heater were excluded from the rest of the analysis.

Proteins showing changes in their production (aeration versus anaerobiosis) comprised glycolytic proteins (glucose-6-phosphate isomerase, galactokinase, glyceraldehyde 3-phosphate dehydrogenase C, UDP-galactopyranose mutase, transketolase, and transaldolase), an enzyme involved in redox reactions (ketol-acid reductoisomerase), and the F_1F_o-ATPase subunit alpha (Table 2).

Changes in gene expression. The expression of the genes coding for some proteins selected from 2DE experiments were further

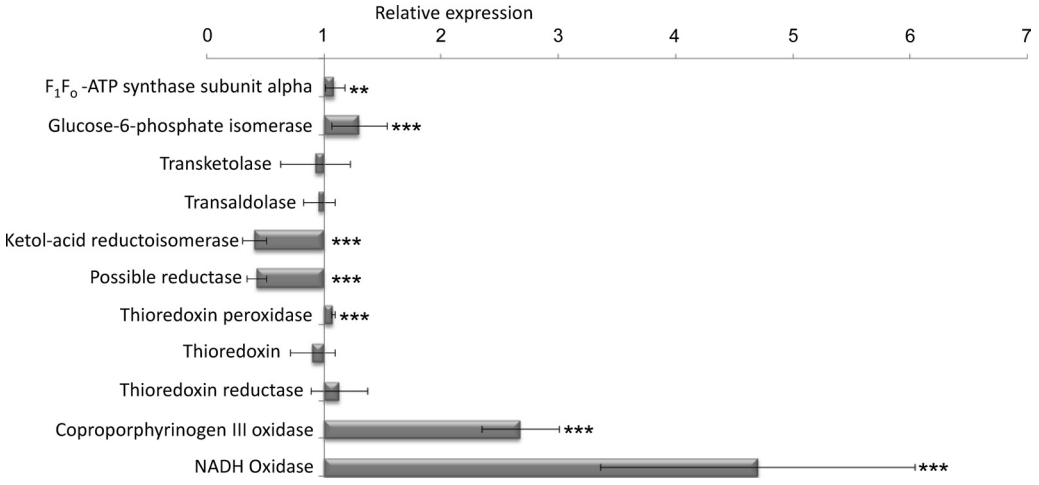

FIG 3 Changes in the relative expression of selected genes as affected by oxygen. In addition, genes whose products may be involved in oxygen detoxification were also included. The results are expressed as means ± standard deviations, and significant differences were assessed by unpaired Student's *t* tests (*, $P < 0.1$; **, $P < 0.05$; ***, $P < 0.001$).

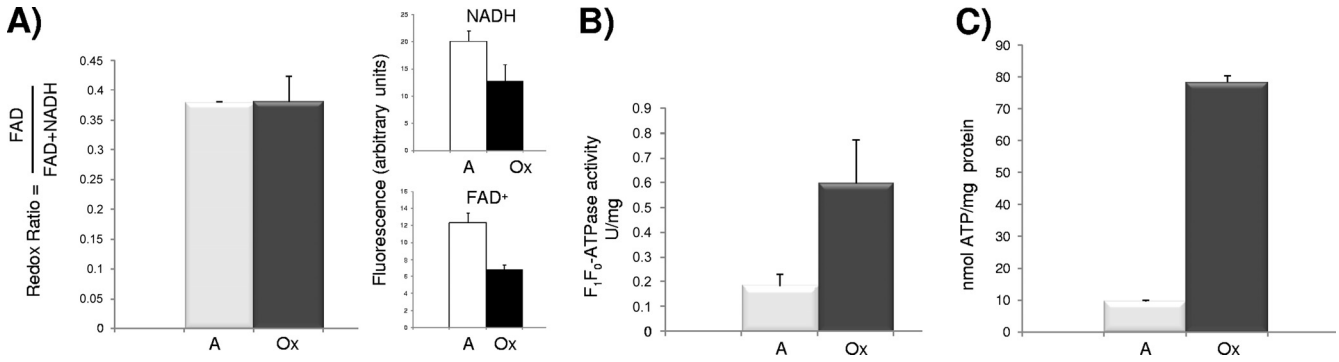

FIG 4 Experiments evidencing changes in different physiological parameters after growth of strain IPLA4549 under anaerobiosis (A) or aeration (Ox). (A) Redox ratios were calculated as the proportion of FAD with respect to the sum of FAD plus NADH fluorescent signals. (B) Specific F_1F_o-ATPase activities in membranes of strain IPLA4549 from cells grown under aeration or anaerobiosis. One unit of F_1F_o-ATPase activity was defined as the amount of protein able to release 1 μmol of inorganic phosphate from ATP hydrolysis in 1 min. (C) Intracellular ATP content, normalized by the concentration of protein. The error bars indicate standard deviations.

analyzed by qRT-PCR (Fig. 3). In this analysis, some putative homologs of genes involved in the processes of tolerance for oxygen and/or oxidative stress in other bacteria (6, 15, 41) were also included (thioredoxin peroxidase, thioredoxin, thioredoxin reductase, coproporphyrinogen III oxidase, NADH oxidase, and possible reductase genes). The genes slightly upregulated by the presence of oxygen were F_1F_o-ATPase subunit alpha, probable UDP-galactopyranose mutase, and thioredoxin peroxidase genes. On the other hand, the genes coding for ketol-acid reductoisomerase and a possible reductase were strongly downregulated by oxygen. The genes showing the greatest increase in their expression were those coding for coproporphyrinogen III oxidase and for a putative NADH oxidase. The first activity catalyzes the oxygen-independent conversion of coproporphyrinogen III to protoporphyrinogen IX, essential for heme biosynthesis and other key redox regulatory targets in bacteria. In contrast, NADH oxidase couples NADH plus H^+ oxidation with a molecule of H_2O_2, rendering two molecules of water. These results prompted us to perform further physiological experiments.

Physiological changes in response to oxygen. According to the changes observed in 2DE and qRT-PCR, different physiological experiments were conducted. First, a redox ratio obtained as described in Materials and Methods from the fluorescent signals of FAD^+ and NADH was computed. As can be seen in Fig. 4A, the redox status of the cells showed no changes, although the individual signals of FAD^+ and NADH showed significant decreases in aeration. On the other hand, higher F_1F_o-ATPase activity was measured in membranes of strain IPLA4549 grown in the presence of oxygen (Fig. 4B). This result was consistent with the higher acetic/lactic acid ratio measured in resting cells (Table 3), in spite of nonsignificant differences in the final carbon balance between the two conditions. This result was also in agreement with the higher intracellular ATP content detected under aerobic conditions (Fig. 4C).

The activities of the two targets showing the highest levels of induction in the qRT-PCR experiments were corroborated by measuring the activities of the corresponding enzymes. First, the activity of coproporphyrinogen III oxidase was higher under aerobic conditions (Fig. 5A). The same was true for the specific activity corresponding to the NADH oxidase in extracts of cells grown under aerobic conditions (Fig. 5B).

DISCUSSION

B. animalis subsp. *lactis* is the most widely used bifidobacterium in dairy products (14). This is mainly due to its good resistance to stressful conditions, including oxygen, as well as bile, acid pH, and heat, with respect to the other bifidobacterial species (32). Little is known about the molecular effectors responsible for the response of bifidobacteria to oxidative damage, with the exception of a few cases, such as a 35.5-kDa protein induced in a *Bifidobacterium longum* aerotolerant strain under aerobic conditions (1). In the present work, we discuss the molecular mechanisms deployed by *B. animalis* subsp. *lactis* IPLA4549 in order to overcome the presence of oxygen during growth.

2DE analysis of cytoplasmic extracts of strain IPLA4549 grown under aeration/anaerobiosis showed that, after aeration, one of the cytoplasmic F_1F_o-ATPase subunits was slightly overproduced. Furthermore, a higher F_1F_o-ATPase specific activity and a higher ATP content were measured under aerobic conditions. ROS have been shown to induce severe cellular damage in DNA, RNA, pro-

TABLE 3 Glucose consumed, acetic and lactic acid production, and acetate/lactic acid ratio in resting cells obtained from IPLA4549 cultures previously grown under aeration or anaerobiosis[a]

Growth condition	Glucose consumed (mM)	Organic acid formation (mM)		Acetic/lactic acid ratio	Carbon balance
		Lactic acid	Acetic acid		
Anaerobiosis	6.74 ± 0.13	4.58 ± 0.07	10.08 ± 0.22	2.20 ± 0.03	0.84 ± 0.02
Aeration	1.34 ± 0.16	0.78 ± 0.07	2.21 ± 0.17	2.67 ± 0.05[b]	0.83 ± 0.05

[a] The results are expressed as concentrations per cell OD_{600} (mM/OD_{600} unit). The carbon balance shown in the last column was calculated from the moles of glucose consumed and the moles of acetic and lactic acid formed.

[b] Significant differences between anaerobiosis and aeration for the physiological parameters considered were established by paired Student's *t* tests. $P < 0.001$.

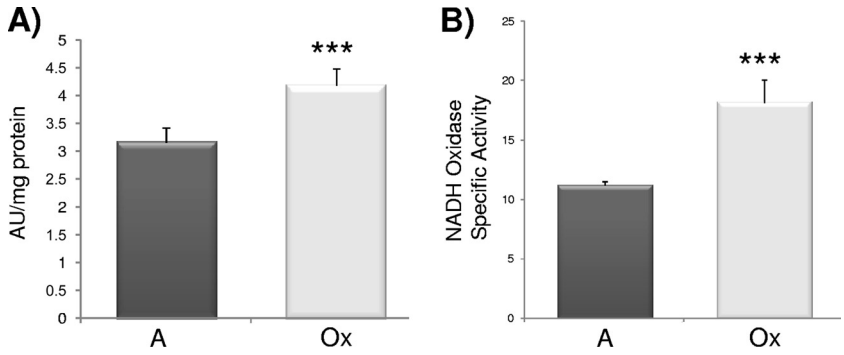

FIG 5 (A) Coproporphyrinogen III oxidase activity measured by a fluorometric determination of the fluorescent substrate protoporphyrin IX. (B) NADH oxidase specific activities calculated from the degradation of NADH followed by absorbance at 340 nm. The data correspond to anaerobiosis (A) and aeration (Ox) conditions. ***, $P < 0.01$. The error bars indicate standard deviations.

tein, and lipids (1). This could suggest that the bacteria may use the ATP surplus generated by F_1F_o-ATPase for fueling enzymes, such as chaperones, oriented to counteract the deleterious effects of oxygen, although this hypothesis must be further investigated.

In addition, several glycolytic proteins were also shown by 2DE to be overproduced. Theoretically, *Bifidobacterium* carries out its glycolytic pathway through the conversion of 2 moles of glucose into 3 moles of acetic acid and 2 moles of lactic acid (34). As mentioned above, the acetate/lactate ratio in strain IPLA4549 increased under aerobic conditions in the absence of significant variations in the carbon balance of the pathway. This was in agreement with previous observations in which the acetate-to-lactate ratio increased in a set of bifidobacterial strains, including *B. animalis* subsp. *lactis*, when grown under aerobic conditions (23, 40). The increased acetic/lactic acid ratio found under aerobic conditions also supports the increased intracellular ATP content observed in strain IPLA4549, since the displacement of the glycolytic pathway toward acetic acid production may theoretically increase ATP production at the substrate level (27).

The acetate-to-lactate ratio is also a parameter related to the redox status of cells. In bifidobacteria, lactate is produced from pyruvate by the action of a NADH-dependent lactate dehydrogenase (27). In the presence of oxygen, the increased amounts of NADH oxidases and peroxidases may compete with lactate dehydrogenase for NADH, thus affecting the formation of end metabolites (39). It has also been observed that elevated levels of intracellular H_2O_2, generated by protonation of superoxide radicals at physiological pH, may block the xylulose-5-phosphate/fructose-6-phosphate phosphoketolase activity, a key enzyme of the bifidobacterial glycolytic pathway (8, 30). Overall, the redox ratios of cell suspensions did not change whether oxygen was present or not, suggesting that the changes observed in genes/enzymes involved in redox reactions may be directed against the negative effects of oxygen in redox metabolites, thus maintaining the redox status of cells.

In this sense, survival in the presence of oxygen has been proposed to be dependent, in *B. longum*, on the presence of NADH oxidases and peroxidases, which might oxidize NADH in the presence of molecular oxygen or H_2O_2, respectively (33). Due to the absence of catalases in the known genome sequences of *B. animalis* subsp. *lactis*, the species must depend on such NADH oxidases and peroxidases in order to detoxify its cytoplasm from oxygen, as happens in *Lactobacillus acidophilus* (7). In this way, the specific activities of both NADH oxidases and peroxidases were notably higher in bifidobacteria grown in the presence of 21% (vol/vol) oxygen (40), and elevated levels of both activities were previously found in some aerotolerant *Bifidobacterium* strains (35). Genes coding for these enzymes seem to be present in all *Bifidobacterium* genomes known so far, although in O_2-sensitive species, the bidirectional reaction catalyzed by NADH oxidases may lead to H_2O_2 accumulation, thus inhibiting the bacterial metabolism (17). No H_2O_2 formation was detected under any of the conditions tested in our study, as already described for aerotolerant *Bifidobacterium* species other than *B. animalis* (15, 16). In turn, higher NADH oxidase specific activity was measured in cells grown under aerobic conditions, a fact that lets us suggest the enzyme as a player involved in detoxification of H_2O_2, a ROS that is accumulated under aerobic conditions.

In addition, coproporphyrinogen III oxidase was upregulated in *B. animalis* subsp. *lactis* IPLA4549. This activity is conducted by two unrelated enzymes, one oxygen dependent and another oxygen independent, leading to oxidation of coproporphyrinogen III to protoporphyrin IX, a key step in heme biosynthesis (6, 20). Higher levels of protoporphyrin IX were captured by oxidation with H_2O_2 under aerobic conditions, suggesting a role for the enzyme in the response of *B. animalis* subsp. *lactis* to oxidative stress. However, further research on the purified coproporphyrinogen III oxidase activity is needed in order to elucidate its precise role in the molecular response of *B. animalis* subsp. *lactis* to oxygen.

Understanding the molecular basis of the stress response is a key factor, not only for the further development of strains with increased stress tolerance, but also to understand the nature of the modifications in cell composition and physiology in order to avoid undesirable changes in the strain properties. The results presented in this paper will help to develop techniques dedicated to preventing oxidative stress in *B. animalis* subsp. *lactis*. This knowledge may lead, in the near future, to the selection of food grade spontaneous mutants able to display improved behavior under aerobic conditions.

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