The activity of the membrane bound H⁺-ATPase of the beer-spoilage bacterium *Lactobacillus brevis* ABBC45 increases upon adaptation to bacteriostatic hop compounds. The ATPase activity is optimal around pH 5.6 and increases up to four fold when *Lb. brevis* was exposed to 666 µM of hop compounds. The extent of activation depends on the concentration of hop compounds and is maximal at the highest concentration tested. The ATPase activity is strongly inhibited by DCCD, a known inhibitor of F₀F₁-ATPase. Western blots of membrane proteins of *Lb. brevis* with the antisera raised against the α- and β-subunits of F₀F₁-ATPase from *Enterococcus hirae* show increased expression of the ATPase after hop adaptation. The expression levels as well as the ATPase-activity decreased to the initial non-adapted levels when the hop-adapted cells were cultured further without hop compounds. These observations strongly indicate that proton pumping by the membrane-bound ATPase contributes considerably to the resistance of *Lb. brevis* to hop compounds.
CHAPTER 6

INTRODUCTION
The hop plant, *Humulus lupulus*, L. is used in beer fermentation for its contribution to the bitter flavor of beer. Furthermore, the usage of hop in the brewing industry is preferred because hop has antibacterial activity and prevents beer from bacterial spoilage. Hop compounds are weak acids, which can cross cytoplasmic membranes in undissociated form in response to the transmembrane pH-gradient (Simpson and Smith, 1992). Due to the higher internal pH these compounds dissociate internally thereby dissipating the pH gradient across the membrane. As a result of this prot onophoric action of hop compounds the viability of the exposed bacteria decreases (Simpson, 1993a, 1993b; Simpson and Smith, 1992). Some bacteria, however, are able to grow in beer in spite of the presence of hop compounds. Sami *et al.* (1997a) reported that *Lactobacillus brevis* ABBC45 strain could adapt to hop treatment and develop a high resistance to hop compounds. During hop resistance development the copy number of plasmid pRH45 harboring *horA* gene increased (Sami *et al.*, 1997a). Subsequent studies revealed that *horA* encodes a bacterial ATP-biding cassette (ABC) multidrug transporter (MDR) which can extrude hop compounds from the cell membranes upon ATP hydrolysis (Sakamoto *et al.*, 2001). As a result of this exogenous expression of HorA in *Lactococcus lactis*, its resistance to hop compounds increased up to two fold. Micro-organisms have been found to increase the proton motive force (pmf)-generating activities in their cytoplasmic membranes when confronted with a high influx of protons (Viegas *et al.*, 1998). The thermophilic bacterium *Bacillus stearothermophilus* (De Vrij *et al.*, 1998) increases proton pumping respiratory chain activities when the proton permeability of its cytoplasmic membrane increases drastically at higher temperatures. In *Enterococcus hirae* (formerly *Streptococcus faecalis*) (Kobayashi *et al.*, 1984, 1986) and *Saccharomyces cerevisiae* (Viegas *et al.*, 1998) the proton translocating ATPase levels in their membranes were found to increase upon exposure to protonophores such as carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP) or weak acids. Obviously, the main reason for this increase of the proton pumping activities is to maintain the pmf and the internal pH at viable levels. In view of the protonophoric activities of hop compounds it was of interest to investigate whether the hop-resistant *Lb. brevis* would respond in a similar way to the action of hop compounds and would increase the functional expression of its proton translocating ATPase in addition to the expression of the MDR HorA. In this study, we demonstrate that this is indeed the case and that *Lb. brevis* increases the functional expression of the proton translocating ATPase during growth in the presence of hop compounds.
MATERIALS AND METHODS

Bacterial strains and growth conditions

*Lactobacillus brevis* ABBC45 was grown anaerobically at 30°C in MRS broth (Merck, Darmstadt, Germany). The initial pH of the growth medium was adjusted to 5.5 with HCl. Hop resistance and expression of HorA was achieved by growth of *Lb. brevis* in the presence of hop compounds, up to 666 µM, as described previously (Sami *et al.*, 1997a). Cells grown in the presence of 666 µM of hop compounds were subcultured without hop compounds added in order to follow the ATPase activity under these growth conditions.

Hop compounds

A concentrated isomerized hop extract (Hopsteiner GmbH, Mainburg, Germany) was used as hop compounds. The iso-α-acid contents were determined, by using high-performance liquid chromatography (HPLC) (Rode *et al.*, 1990). The concentration of hop compounds in the medium was expressed as the concentration of iso-α-acids.

Preparation of the membrane

*Lb. brevis* was grown to late exponential phase in the absence and in the presence of 100 µM and 666 µM hop compounds. Cells of *Lb. brevis* were harvested by centrifugation at 7,000 × g for 15 min and washed twice at room temperature in 50 mM (K) HEPES (pH 7.4) containing 5 mM MgSO₄. The cells, suspended in the same buffer, were lysed at 37°C by treatment for 1.5 h with 1 mg/ml lysozyme (Sigma, USA) and 50 µg/ml mutanolysin (Sigma, USA) in the presence of a cocktail of proteinase inhibitors (Complete® [Boehringer Mannheim, Germany]). After the addition of DNase I (50 µg/ml) and RNase (1 µg/ml), the suspension was passed three times through an ice-cold French pressure cell at 70 MPa. Unbroken cells were subsequently removed by centrifugation at 7,000 × g for 15 min at room temperature. The supernatant was centrifuged at 200,000 × g for 45 min at 4°C and the pellet was suspended in the same buffer. This membrane fraction was used for ATPase assays and Western Blot analysis. The concentration of the membrane proteins was determined with D C protein assay kit (BioRad, USA) with bovine serum albumin as a quantitative standard.

ATPase Assay

ATPase activity was estimated from the release of inorganic phosphate measured by a modification of the method of Driessen *et al.* (1991). 1 or 2 µg of membrane protein was incubated at 30°C for 10 min in 50 mM (K) Mes buffer (usually at pH 5.5) containing 5 mM MgCl₂. ATP [Potassium salt] was added at a final
concentration of 2 mM to initiate the reaction. The reaction (total volume of 40 µl) was stopped after 5 min by immediately cooling the test tubes on ice. Malachite green solution (200 µl of 0.034%) was added, and after 40 min the color development was terminated by the addition of 30 µl citric acid solution (34% [w/v]). Immediately, the absorbance at 660 nm was measured with a multiscan photometer (Multiskan MS; Labsystems, Finland). One unit ATPase-activity was defined as the release of 1 µmole of inorganic phosphate in 1 min. Calibration was done by using a series of Pi standards (Sigma, USA). For the determination of pH dependency of the ATPase activity, membranes were incubated for 60 min on ice in 50 mM (K) Mes buffer, adjusted to various pH values. The ATPase activity was assayed at those different pH values as described above. To measure the effects of inhibitors on the ATPase activity, the membranes were pre-incubated with N, N’-dicyclohexylcarbodiimide (DCCD; final concentration 0.2 mM), ortho-vanadate (final concentration 0.2 mM) or nitrate (K2NO3; final concentration 25 mM) for 10 min at 30°C, and subsequently for 60 min on ice. The membrane sample without inhibitor was used as the control.

**Western Blotting Analysis**

The membrane protein of *Lb. brevis*, prepared as described above, was solubilised in Laemmli sample buffer containing 2% SDS (Laemmli, 1970) and separated by electrophoresis (20 µg of protein / lane) through 10% SDS-polyacrylamide gel by the method of Laemmli (1970). The protein bands were transferred to a polyvinilidene difluoride (PVDF) filter membrane and detected with the antisera raised against the F1 complex of *Enterococcus hirae* H+-ATPase (Arikado *et al.*, 1999) which can also bind with F0F1-ATPase from *Lactococcus lactis* (Amachi *et al.*, 1998). Membranes from *E. hirae* prepared as previously described (Arikado *et al.*, 1999) were used as control. The antibody-bound proteins were made visible with nitrobluetertazorium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Gibco BRL. USA). The intensities of the bands were measured by densitometric analysis with NIH Image software v.1.61 (NIH, USA).
RESULTS

Effect of hop on the ATPase activity

In a previous publication it has been demonstrated that under these conditions \textit{Lb. brevis} develops hop resistance by overexpressing the MDR HorA (Sami, 1999). The cytoplasmic membranes of cells were isolated as described in Materials & Methods and the ATPase activities in these membranes were determined as a function of the pH ranging from pH 4.4 to 7.0. All membranes of \textit{Lb. brevis} grown in the presence of different levels of hop compounds showed maximum ATPase activity at around pH 5.6 (Fig. 1). At pH 5.6 membranes from the cells adapted to 666 µM of hop compounds had the highest activity, which was about 4-times the ATPase activity of membranes from non-adapted cells. The ATPase activities of membranes from cells adapted to 100 µM of hop compounds were in between these extremes and about 1.7 times the activity of the membranes from the non-adapted cells. Once the hop-adapted cells (666 µM) were subcultured in medium without hop compounds the ATPase activity of their membranes decreased rapidly (Fig. 1).

![Figure 1. The pH profile of the ATPase activity in membranes of \textit{Lb. brevis}.](image)

The ATPase activity at pH values ranging from 4.4 to 7.0 was measured of membranes prepared from cells grown without hop compounds (W0, ○) and of cells adapted to 100 µM (W100, ×) and to 666 µM of hop compounds (R666, ■), and of cells de-adapted by growth first in the presence of 666 µM of hop compounds followed by growth for two days in the absence of hop compounds (R0, ▲). The ATPase activity was shown as the amount of released inorganic phosphate (Pi) per min / mg protein.

Effect of inhibitors on the ATPase activity

To characterize the type of ATPase present in the membrane of \textit{Lb. brevis}, the effect of several kinds of inhibitors on the ATPase activity was studied (Fig. 2). The ATPase activities of membranes from non-adapted cells and from cells adapted to different concentrations of hop compounds were all significantly inhibited by the F$_0$F$_1$-type inhibitor \textit{N, N'}-dicyclohexylcarbodiimide (DCCD). Moderate inhibition was observed with the P-type inhibitor ortho-vanadate, while the V-type inhibitor K$_2$NO$_3$ showed the least inhibition or even activation in
membranes from cells grown at 100 µM of hop compounds (W100 in Fig. 2). These results correspond to the observations made for the enterococcal F₀F₁-type ATPase, which is slightly inhibited by ortho-vanadate and slightly enhanced by K₂NO₃ (Y. Kakinuma, personal communication), indicating that F₀F₁-type ATPase is the major ATPase in membranes from *Lb. brevis*.

![Figure 2. The effect of inhibitors on the ATPase activity of *Lb. brevis.*](image)

**Western Blot Analysis**

Two bands were strongly detected from the membranes of *Lb. brevis* with the antisera against the α- and β- subunits of the F₁ ATPase complex from *E. hirae*, which strongly indicates the F₀F₁-type nature of the ATPase of *Lb. brevis*. The apparent molecular weights of these bands are slightly higher than those of the α- and β- subunits of F₁ from *E. hirae* (Fig. 3A). The intensities of both bands are higher in membranes isolated from cells grown at higher concentrations of hop compounds and decrease again in membranes from hop-adapted cells (666 µM) subcultured in medium without hop compounds (Fig. 3B.). The intensities of both bands correlated well (r = 0.990; r = correlation coefficient) with the ATPase activities of the different membranes. The rate and extent of growth in MRS broth of hop adapted cells are slower than of non-adapted cells (Sami *et al.*, 1997a). Also hop-adapted cells are smaller than cells grown in the absence of hop compounds (data not shown).
Figure 3. Western blot analysis of membranes of *Lb. brevis* and *E. hirae* with antisera against F1 of *E. hirae*. Membranes of *Lb. brevis* were solubilized and separated by electrophoresis through 10% polyacrylamide gel (lanes 2-5). For comparison the results with membranes from *E. hirae* are shown in lane 1. The proteins were transferred to a PVDF filter membrane and reacted with the antisera raised against the F1 complex of *E. hirae* H⁺-ATPase. (A) The result of Western Blotting: Lanes 1, *E. hirae* cultured at pH 6.0; Lane 2, *Lb. brevis* grown without hop (W0); Lane 3, *Lb. brevis* adapted to 100 µM of hop compounds (W100); Lane 4, *Lb. brevis* adapted to 666 µM of hop compounds (R666); Lane 5, *Lb. brevis* de-adapted from 666 µM to 0 µM of hop compounds (R0). The arrows indicated the position of the α- or β-subunit of H⁺-ATPase from *E. hirae*. (B) The intensity of the lower bands of the ATPase from *Lb. brevis*. The intensities of these bands were measured with NIH Image software and presented in arbitrary units.

DISCUSSION

The beer spoilage bacterium *Lb. brevis* ABBC45 develops hop resistance upon growth in hop-containing media (Sami *et al.*, 1997a). This resistance was found to be mediated by the functionally expressed multidrug resistance ABC transporter HorA (Sami, 1999; Sakamoto *et al.*, 2001). Studies of HorA, functionally expressed in *Lactococcus lactis*, revealed that HorA can excrete the lipophilic hop compounds and several other MDR substrates from the membrane into the external medium (Sakamoto *et al.*, 2001). Recently a second proton motive force-driven MDR with affinity for hop compounds has been found in *Lb. brevis* ABBC45 lacking HorA (Suzuki *et al.*, 2002). The activity of HorA and this pmf-driven MDR thus results in a reduced influx of the undissociated and membrane permeable iso-α-acids into the cytoplasm and thereby limits the anti-bacterial pmf-dissipating effect of hop compounds. Since *Lb. brevis* develops resistance against rather high concentrations of hop compounds, the question arose whether
functional expression of HorA and the pmf-driven MDR was sufficient to confer this resistance or whether additional activities could contribute to hop resistance. Anaerobic Gram-positive lactic acid bacteria such as *Lb. brevis* depend for the generation of their proton motive force strongly on their membrane bound H⁺-F₀F₁-ATPase (Kobayashi et al., 1986; Konings et al., 1995). In this study, we demonstrated that the functional expression of a membrane-bound H⁺-F₀F₁-ATPase increased during hop-resistance development and decreased again when the exposure to hop compounds was stopped. Previously, it was demonstrated that also the expression of the HorA transporter increased during hop-resistance development (Sami, 1999). The H⁺-F₀F₁-type nature of the ATPase was confirmed by H⁺-F₀F₁-ATPase effectors and especially by immunological studies with the antisera against α- and β-subunits of H⁺-F₀F₁-ATPase from *E. hirae*. In accordance with the observations of Kobayashi et al. (Kobayashi et al., 1984, 1986) made in the anaerobic Gram-positive bacterium *E. hirae*, the increased functional expression of H⁺-F₀F₁-ATPase most likely allows *Lb. brevis* to maintain a viable pmf and intracellular pH in the presence of the protonophoric hop compounds.

The results of this study together with those of previous reports (Sami, 1999; Sakamoto et al., 2001; Suzuki et al., 2002) indicate that *Lb. brevis* becomes resistant to hop compounds by the combined action of two ATP-driven systems: the H⁺-ATPase and the MDR pump HorA (Sami, 1999; Sakamoto et al., 2001), and a pmf-driven MDR (Suzuki et al., 2002). HorA and the pmf-driven MDR reduce the influx of the weak acidic hop compounds by pumping the undissociated hop compounds from the membrane environment into the external medium. The H⁺-ATPase compensates for the pmf-dissipating and internal pH-decreasing effects of hop compounds, which have escaped the MDR activities, by pumping more protons from the cytoplasm across the membrane. As a result of the higher expression of ATPase and of HorA and the energy dissipation by hop compounds the rate and extent of growth in MRS broth of hop adapted cells are slower than of non-adapted cells (Sami et al., 1997a). Those various hop resistance mechanisms (Fig. 4) are another demonstration of the versatility of bacteria and their capacity to recruit a variety of mechanisms to cope with toxic compounds in their environments.
Figure 4. Proposed mechanisms of hop resistance in *Lb. brevis* ABBC45 by the combined action of two ATP-driven systems and one proton motive force-driven MDR. The undissociated hop compounds (Hop-H) intercalate into the cytoplasmic membrane and are pumped out by the multidrug resistant ABC-type transporter HorA (a) (Sami, 1999; Sakamoto *et al.*, 2001) and by a secondary MDR (b) (Suzuki *et al.*, 2002). A fraction of Hop-H escapes the pumping activity of the transporters and enters the cytoplasm. In the cytoplasm Hop-H dissociates due to the higher internal pH into the anion (Hop-) and H+. H+ also enters the cytoplasm in antiport with Hop-H by the secondary transporter. Hop- may bind to cations such as Mn2+ (Archibalt and Fridovich, 1981; Simpson, 1993a, 1993b; Simpson *et al.*, 1993: Simpson and Hughes, 1993), while the increased H+-ATPase activity excretes H+ across the membrane (c) (This work).