SUMMARY

Beer has a long history of 5,000-7,000 years. Since the start of beer production, brewers have been bothered by beer spoilage. The introduction of hop compounds from the hop plant, *Humulus Lupulus*, L., into beer in the 12th to 13th century was a major breakthrough due to the strong preservative value of hops. Nevertheless several microorganisms can still grow in beer. These beer spoilage microorganisms include a few lactic acid bacteria, a few Gram-negative bacteria and wild yeasts. Beer spoilage lactic acid bacteria include *Lactobacillus* and *Pediococcus* species and are the major contaminants in the brewing industry. They spoil beer by producing acidity, turbidity and, in some species, ropiness and a buttery off-odor of diacetyl. Aerobic acetic acid bacteria such as *Acetobacter* and *Gluconobacter* species were well-known beer spoilage Gram-negative bacteria. However, these aerobes have been replaced in the past decades by strictly anaerobic Gram-negative bacteria such as *Pectinatus* and *Megasphaera* species, due to the drastically reduced oxygen content in beer by the improved brewing technology. These anaerobic Gram-negatives cause more serious spoilage than lactic acid bacteria by producing an offensive rotten egg smell of hydrogen sulfide. Wild yeasts are mainly detected at taps and dispense lines at pubs but rarely in packaged beer. These yeasts cause less serious spoilage problems than bacteria.

Detection and identification of beer spoilage micro-organisms are very important for the quality assurance in breweries. The most popular method today is still the conventional culture method. It can take a week or even longer to detect microorganisms and consequently the products are often already released for sale before the microbiological results become available. Hence more rapid detection methods for beer spoilage bacteria are required. A very powerful tool is the polymerase chain reaction (PCR) since it enables detection and identification of micro-organisms in a very short period of time. Sets of PCR primers targeting species-specific regions in the bacterial 16S ribosomal RNA gene (rDNA) have been developed for each of the known beer spoilage bacteria (Chapter 2). However false negative results were sometimes obtained as PCR can be prevented by the presence of factors such as polyphenols in beer samples. These false negatives can be eliminated by the use of the primers targeting to consensus sequences in bacterial 16S rDNA as an internal positive control for proper PCR (Chapter 2).

Most strains in *Lactobacillus brevis* and *Pediococcus damnosus* can grow in beer, but some can not. For the quality assurance discrimination of beer spoiling strains from non-spoilers is therefore necessary. The most crucial feature in beer spoilage strains of lactic acid bacteria is resistance to hop compounds present in beer as iso-α-acids. These hop compounds can inhibit the growth of Gram-positive bacteria. The mechanism of the antibacterial action of hop compounds was studied. The
major component of iso-α-acids, trans-isohumulone, was found to function as an ionophore in a hop sensitive strain of *Lb. brevis* and to catalyze electroneutral exchange across the cytoplasmic membrane of protons for intracellular cations such as Mn^{2+} (Simpson, 1993b). Consequently the electrochemical proton gradient across the cytoplasmic membrane is dissipated, resulting in the decrease of proton motive force (pmf). The uptake of nutrients by pmf-driven uptake systems will then also be decreased. Resistance to hop compounds in *Lb. brevis* has been studied at the molecular level. The *horA* gene, encoding a polypeptide that is 53% identical to LmrA, a lactococcal ABC-type multidrug transporter (van Veen et al., 1996), was discovered in a plasmid pRH45 of the hop-resistant strain of *Lb. brevis* ABBC45 (Sami et al., 1997a). Amplification of pRH45 occurs when this strain is grown in a continuous culture with increasing concentration of iso-α-acids. On the other hand, hop resistance decreased significantly when *Lb. brevis* ABBC45 was cured from pRH45, and resistance was regained when this plasmid was re-introduced by electrottransformation (Chapter 3 and 4). HorA was successfully expressed in *Lactococcus lactis* under control of the nicin-inducible expression system (Chapter 5). Studies in cells, membrane vesicles and proteoliposomes, reconstituted with solubilized and purified HorA, revealed that this protein confers hop resistance by excreting hop compounds in an ATP-dependent manner from the cell membrane to outer medium. In addition to the activity of HorA also increased proton pumping by the membrane bound H^{+}-ATPase contributes to increased hop resistance (Chapter 6). To energize such ATP-dependent transporters hop resistant cells contain larger ATP pools than hop sensitive cells (Simpson and Fernandez, 1994). Furthermore evidence for the presence of a proton motive force dependent hop transporter was recently presented (Suzuki et al., 2002). The potential molecular mechanisms of hop resistance in *Lb. brevis* are shown in Fig. 1.

Understanding the mechanisms of hop resistance has enabled the development of rapid methods to discriminate beer spoilage strains from non-spoilers. The *horA*-PCR method in which a set of specific primers detects the *horA* gene or its homologues has been applied for bacterial control in breweries (Sami et al., 1997b). Most *horA* positive strains of *Lactobacillus* spp. were found to have beer spoiling ability. Also a discrimination method was developed based on ATP pool measurement in lactobacillus cells (Okazaki et al., 1997). However, some potential hop resistant strain cannot grow in beer when they have not first been exposed to sub-inhibitory concentration of hop compounds (Simpson and Fernandez, 1992).

The beer spoilage ability of *Pectinatus* spp. and *Megasphaera cerevisiae* has been poorly studied. Since all strains have been reported to be capable of beer spoiling, species identification is sufficient for the brewing industry. However, with the current trend of beer flavor (lower alcohol and bitterness), there is the potential risk that not yet reported bacteria will contribute to beer spoilage. Investigation of the beer spoilage ability of especially Gram-negative bacteria may be useful to
reduce this risk.

**Figure 1. Mechanisms of hop resistance.** Hop compounds act as ionophores that exchange protons for cellular divalent cations. In a hop-sensitive cell, hop compounds (Hop-H) invade the cell and dissociate into hop anions and protons due to the higher internal pH. Hop anions trap divalent cations such as Mn$^{2+}$ and diffuse out of the cell. The ionophoric action together with the diffusion of the hop-metal complex results in an electroneutral exchange of cations. Release of protons from hop compounds decreases the intracellular pH and results in a dissipation of the transmembrane proton gradient (∆pH) and the proton motive force (pmf). Consequently, pmf-driven uptake of nutrients will be decreased. In hop resistant cells hop compounds can be expelled from the cytoplasmic membrane by HorA (a) (Sakamoto et al., 2001) and probably also by a pmf-dependent transporter (b) (Suzuki et al., 2002). Furthermore, overexpressed H$^{-}$-ATPase increases the pumping of protons released from the hop compounds (c) (Sakamoto et al., 2002). More ATP is generated in hop-resistant cells than in hop-sensitive cells (Simpson and Fernandez, 1994). Galactosylated glycerol teichoic acid in the cell wall (Yasui et al., 1997) and a changed lipid composition of the cytoplasmic membrane of beer spoilage lactic acid bacteria may increase the barrier to hop compounds.