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## Beer spoilage bacteria and hop resistance in *Lactobacillus brevis*

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# CHAPTER 1

-Review-

## Beer Spoilage Bacteria and Hop Resistance

Kanta Sakamoto and Wil N. Konings

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## CHAPTER 1

### 1. INTRODUCTION

Beer has been recognized for hundreds of years as a safe beverage. It is hard to spoil and has a remarkable microbiological stability. The reason is that beer is an unfavorable medium for many micro-organisms due to the presence of ethanol (0.5-10% w/w), hop bitter compounds (approx. 17-55 ppm of iso- $\alpha$ -acids), the high content of carbon dioxide (approx. 0.5% w/v), the low pH (3.8-4.7), the extremely reduced content of oxygen (<0.1 ppm) and the presence of only traces of nutritive substances such as glucose, maltose and maltotriose. These latter carbon sources have been substrates for brewing yeast during fermentation. As a result pathogens such as *Salmonellae typhimurium* and *Staphylococcus aureus* do not grow or survive in beer (Bunker, 1955).

However, in spite of these unfavorable features a few micro-organisms still manage to grow in beer. These, so-called beer spoilage microorganisms, can cause an increase of turbidity and unpleasant sensory changes of beer. Needless to say that these changes can affect negatively not only the quality of final product but also the financial gain of the brewing companies.

A number of micro-organisms have been reported to be beer spoilage micro-organisms, among which both Gram-positive and Gram-negative bacteria, as well as so-called wild yeasts. Gram-positive beer spoilage bacteria include lactic acid bacteria belonging to the genera *Lactobacillus* and *Pediococcus*. They are recognized as the most hazardous bacteria for breweries since these organisms are responsible for approximately 70% of the microbial beer-spoilage incidents (Back, 1994). The second group of beer spoilage bacteria is Gram-negative bacteria of the genera *Pectinatus* and *Megasphaera*. The roles of these strictly anaerobic bacteria in beer spoilage have increased since the improved technology in modern breweries has resulted in significant reduction of oxygen content in the final products. Wild yeasts do cause less serious spoilage problems than bacteria but are considered a serious nuisance to brewers because of the difficulty to discriminate them from brewing yeasts.

Considerable effort has been made by many microbiologists to control microbial contamination in beer. The most commonly used method today for detecting beer spoilage micro-organisms in breweries is still traditional incubation on culture media. A number of selective media have been developed since Louis Pasteur published in 1876 'Études sur La Bière (Studies on beer)'. It usually takes a week or even longer for bacteria to form visible colonies on plates or to increase the turbidity in nutrient broths. Consequently, the products are often already released for sale before the microbiological results become available. If a beer spoilage micro-organism is then detected and identified in the beer product it needs to be recalled from the market. This will cause serious commercial damages to the brewery. Most microbiologists have focused on developing more specific and rapid methods for the detection of beer spoilage micro-organisms than using the

traditional culture methods. A number of advanced biotechnological techniques are employed such as immunoassays with antibodies specific to beer spoilage bacteria. Recently polymerase chain reaction (PCR) technology, targeting specific nucleotide sequences of ribosomal RNA genes (rDNA), has been successfully applied for the rapid identification of both beer spoilage bacteria and wild yeasts. These methods can identify exactly the taxonomy of the micro-organism(s) found in beer. However, not all beer spoilage bacteria can actually grow in beer. A method is needed to determine whether the detected bacterium is capable of growing in beer or not. Up to date the only available method is the so-called 'forcing test' in which the detected bacterium is re-inoculated and incubated in beer. It usually takes one month or even longer to detect visible turbidity in the inoculated beer, meaning that this method is not very practical. A rapid method to predict beer spoiling ability is therefore urgently needed and the development of such a method will be essential for understanding the nature of the beer-spoiling ability.

Among the components of beer, hop compounds have received a lot of attention for reason of their preservative values and their bitterness. For centuries it was generally believed that hops protect beer from infection by most organisms, including pathogens, but it was only in the 20th century that Shimwell (1937a, 1937b) showed that hop compounds only inhibit growth of Gram-positive bacteria and not of Gram-negative bacteria. His findings had a great impact because many pathogens such as *Salmonella* species are Gram-negative bacteria. Feature(s) such as low pH and alcohol content undoubtedly have a negative effect on growth of these pathogens in beer. Among Gram-positive bacteria some species of lactic acid bacteria are less sensitive to hop compounds and are able to grow in beer. Insight in the mechanism of resistance of lactic acid bacteria to hop compounds is crucial for understanding their beer spoiling ability. The antibacterial activity of hop compounds and the hop-resistance of lactic acid bacteria have extensively been investigated. On the other hand little studies have been done on the beer spoiling ability of Gram-negative bacteria.

This chapter reviews the currently available information about beer spoilage bacteria, their growth and spoilage activity in beer.

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### 2. BEER SPOILAGE BACTERIA

Beer is a poor and rather hostile environment for most micro-organisms. Its ethanol concentration ranges from 0.5 to 10% (w/w) and is usually around 4 to 5%. These concentrations are high enough to make beer bacteriostatic or bactericidal. Beer is usually slightly acid with pH's ranging from pH 3.8 to 4.7, which is lower than most bacteria can tolerate for growth. Furthermore, the high carbondioxide concentration (approx. 0.5% w/v) and extremely low oxygen content (<0.1 ppm) makes beer a near to anaerobic medium.

Beer also contains bitter hop compounds (approx. 17-55 ppm of iso- $\alpha$ -acids), which are toxic, especially for Gram-positive bacteria. The concentrations of nutritive substances, such as saccharides and amino acids, are very low since most have been consumed by brewing yeasts during fermentation.

Only a few bacteria are able to grow under such inhospitable conditions and are able to spoil beer (see Table 1). These bacteria include both Gram-positive and -negative species. Gram-positive beer spoilage bacteria belong almost always to the lactic acid bacteria. They are regarded as most harmful for brewing industry and are the cause of most of bacterial spoilage incidents. Only a few Gram-negative bacteria are known to cause beer spoilage. Some of these belong to the acetic acid bacteria and have received most attention. Today these aerobic bacteria do not present a serious problem in beer spoilage anymore, since improved brewing technology has led to a drastic reduction of the oxygen content in beer. Instead strictly anaerobic bacteria, typically *Pectinatus* spp. and *Megasphaera cerevisiae*, have become serious beer spoilage bacteria.

#### 2.1. Gram-Positive Bacteria

Almost all the beer spoilage Gram-positive bacteria belong to lactic acid bacteria. They are a large group of species and genera of Gram-positive bacteria. Only a few of these lactic acid bacteria are beer-spoiling organisms. Most hazardous for the brewing industry are those belonging to the genera *Lactobacillus* and *Pediococcus*. In the period 1980-1990, 58-88% of the microbial beer-spoilage incidents in Germany were caused by lactobacilli and pediococci (Back *et al.*, 1988; Back, 1994). Also in Czech all beer-spoilage bacteria detected in the breweries belonged to lactic acid bacteria (Hollerová and Kubizniaková, 2001). The situation in other countries seems to be similar although for commercial reasons little statistical information has been supplied. These lactic acid bacteria spoil beer by producing haze or rope and cause unpleasant flavor changes such as sourness and atypical odor.

##### 2.1.1. Lactobacilli

The genus *Lactobacillus* is the largest genus of lactic acid bacteria and includes numerous species. They are widely used in various fermentation processes,

including food products such as beer, wine, yoghurt and pickles. In contrast to the general believe that all lactobacilli can grow in beer, only a few species have been reported to be capable of beer spoilage (Rainbow, 1981; Priest, 1987, 1996; Jespersen and Jakobsen, 1996). *Lb. brevis* appears to be the most important beer spoiling *Lactobacillus* species and is detected at high frequency in beer and breweries. More than half of the bacterial incidents were caused by this species (Back *et al.*, 1988; Back, 1994; Hollerová and Kubizniaková, 2001). *Lb. brevis* is an obligate heterofermentative bacterium. It is one of the best-studied beer spoilage bacteria and grows optimally at 30°C and pH 4-6 and is generally resistant to hop compounds. It is physiologically versatile and can cause various problems in beer such as super-attenuation, due to the ability to ferment dextrans and starch (Lawrence, 1988). The antibacterial effects of hop compounds and the mechanism(s) responsible for hop resistance have been studied in detail in this species (Simpson, 1991, 1993a, 1993b; Simpson and Smith, 1992; Fernandez and Simpson, 1993; Simpson and Fernandez, 1994; Sami *et al.*, 1997a, 1997b, 1998; Sakamoto *et al.*, 2001, 2002; Suzuki *et al.*, 2002). These studies will be described in Chapter 4, 5 and 6.

The second most important beer spoiling lactobacillus, *Lb. lindneri* is responsible for 15-25% of beer-spoilage incidents (Back *et al.*, 1988; Back, 1994). The physiology of *Lb. lindneri* is very similar to that of *Lb. brevis* and only recently has *Lb. lindneri* been recognized on the basis of its 16S rRNA gene sequence as a phylogenetically separate species in the genus *Lactobacillus* (Back *et al.*, 1996; Anon., 1997). *Lb. lindneri* is highly resistant to hop compounds (Back, 1981) and grows optimally at 19-23°C (Priest, 1987; Back *et al.*, 1996) but survives higher thermal treatments than other lactic acid bacteria (Back *et al.*, 1992). All *Lb. lindneri* strains, tested so far, are capable of beer spoilage, while other lactobacilli comprise both beer spoiling and non-spoiling strains (Rinck and Wackerbauer, 1987a, 1987b; Storgårds *et al.*, 1998). It is particularly problematic that *Lb. lindneri* grows slowly on media commonly used in breweries while growth in beer can be very rapid.

*Lb. buchneri*, *Lb. casei*, *Lb. coryneformis*, *Lb. curvatus* and *Lb. plantarum* are less common beer spoiling bacteria than the two species described above (Priest, 1996). *Lb. buchneri* resembles closely *Lb. brevis*, but differs in its ability to ferment melezitose. In addition, some *Lb. buchneri* strains require riboflavin in their growth medium (Sharpe, 1979; Back, 1981). *Lb. casei* can produce diacetyl, which gives beer an unacceptable buttery flavor. Diacetyl appears to be more potent in that respect than lactic acid, the major end-product of lactic acid bacteria. The threshold value of diacetyl in beer is much lower (0.15 ppm) than of lactic acid (300 ppm) (Hough *et al.*, 1982). Diacetyl is also produced during normal fermentation by yeast and too high levels of diacetyl are produced when yeast is not properly removed from young beer (Inoue, 1981). *Lb. casei* is particularly problematic

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because its contamination in finished beer can give rise to high levels of diacetyl. The pathway of diacetyl formation by lactic acid bacteria has been studied in detail (Speckman and Collins, 1968, 1973; Jönsson and Pettersson, 1977).

*Lb. brevisimilis* (Back, 1987), *Lb. malefermentans*, *Lb. parabuchneri* (Farrow *et al.*, 1988), *Lb. collinoides* and *Lb. paracasei* subsp. *paracasei* (Hollerová and Kubizniaková, 2001) have also been reported to be beer spoilage species.

Recently few additional newly discovered species of lactobacilli have been added to the list of beer spoilers. According to its 16S rRNA gene sequence, a strain called BS-1 is related to *Lb. coryneformis*, but its narrow fermentation pattern (only limited to glucose, mannose and fructose) differs significantly not only from that of *Lb. coryneformis* but also from other *Lactobacillus* spp. (Nakakita *et al.*, 1998). Two other novel lactobacillus species were found in spoiled beer with significantly different taxonomical properties from those of other *Lactobacillus* spp. One species, LA-2, with a 16S rRNA gene sequence 99.5% similar to that of *Lb. collinoides*, has a strong beer spoiling ability. The other species, LA-6, has a weak beer spoiling ability and did not show any significant homology to the other *Lactobacillus* spp. (Funahashi *et al.*, 1998).

### 2.1.2. Pediococci

Pediococci are homofermentative bacteria which grow in pairs and tetrads. They were originally known as 'sarcinae' because their cell organization was confused with that of true sarcinae. Beer spoilage, caused by cocci and characterized by acid formation and buttery aroma of diacetyl, was therefore called 'sarcina sickness'. *Pediococcus* spp. produce rope, and extensive amounts of diacetyl like *Lb. casei*. They are found at many stages in the brewing process from wort till finished beer. Several *Pediococcus* spp. have been found in breweries: *P. acidilactici*, *P. damnosus*, *P. dextrinicus*, *P. halophilus* (recently classified as *Tetragenococcus halophilus*), *P. inopinatus*, *P. parvulus* and *P. pentosaceus* (Back, 1978; Back and Stackebrandt, 1978). Among them is *P. damnosus* the most common beer spoiler. It was responsible for more than 20% of all bacterial incidents in the period 1980-87 (Back *et al.*, 1980, 1988) but only for 3-4% in 1992-93 (Back, 1994). The incidence of beer spoilage by pediococci has decreased most likely as a result of improved sanitation conditions in breweries. *P. damnosus* is generally resistant to hop compounds. It is interesting that *P. damnosus* is commonly found in beer and late fermentations, but seldom in pitching yeast. In contrast *P. inopinatus* is frequently detected in pitching yeast but rarely in the other stages of beer fermentations (McCaig and Weaver, 1983; Priest, 1987). *P. inopinatus* and *P. dextrinicus* can grow in beer at pH values above 4.2 and with low concentrations of ethanol and hop compounds (Lawrence, 1988). *P. pentsauces* and *P. acidilactici* have never been reported to cause any defect in finished beer (Simpson and Taguchi, 1995). The amount of diacetyl produced by pediococci varies from

species to species. *P. damnosus* produces large amounts of diacetyl, *P. inopinatus* less and *P. pentsauces* not at all. Brewers therefore usually pay attention only to *P. damnosus*.

### 2.1.3. Other Gram-Positive Bacteria

In addition to *Lactobacillus* and *Pediococcus* species also a species from the genus *Microcococcus* has been reported to be occasionally responsible for beer spoilage. *M. kristinae* can grow in beer with low ethanol and hop compounds at pH values above 4.5 (Back, 1981). Micrococci are usually strictly aerobic, but *M. kristinae* can grow also under anaerobic condition (Lawrence and Priest, 1981). It produces a fruity atypical aroma in beer (Back, 1981).

## 2.2. Gram-Negative Bacteria

Several genera of Gram-negative bacteria are known to be involved in beer spoilage. The presence of a hydrophobic outer membrane makes Gram-negative bacteria generally resistant to hop compounds. Aerobic acetic acid bacteria i.e. *Gluconobacter* and *Acetobacter* spp. were well-known as beer spoilage organisms in breweries but the role of these bacteria in beer spoilage has been reduced significantly due to the much lower oxygen content during the brewing processes and in packaged beer of modern breweries. Instead, the occurrence of strictly anaerobic bacteria in beer spoilage incidents has increased. These include the genera *Pectinatus*, *Megasphaera*, *Selenomonas*, *Zymomonas* and *Zymophilus*. Especially *Pectinatus* and *Megasphaera* species cause much more serious problems for breweries than lactobacilli and pediococci, mainly due to the production of the offensive ‘rotten egg’ odor in finished beer.

### 2.2.1. Pectinatus

*Pectinatus* spp. are now recognized as one of the most dangerous beer spoilage bacteria. They play a major role in 20 to 30% of bacterial incidents, mainly in non-pasteurized beer rather than in pasteurized beer (Back, 1994). *Pectinatus* species were long thought to be *Zymomonas* spp. because of their phenotypical similarities. The first isolate was obtained from breweries in 1971 (Lee *et al.*, 1978) and so were all subsequent isolates (Back *et al.*, 1979; Haukeli, 1980; Kirchner *et al.*, 1980; Haikara *et al.*, 1981; Takahashi, 1983, Soberka *et al.*, 1988, 1989). The natural habitat of the *Pectinatus* species are still unknown (Haikara, 1991). Two species are found in this genus: *P. cerevisiiphilus* and *P. frisingensis* (Schleifer *et al.*, 1990). There is also one strain DSM20764 isolated from spoiled beer that differs considerably in genotype from the two other species (Weiss, personal communication, 1987). Its 16S rRNA gene sequence is distinctly different from that of the other two species (Sakamoto, 1997). *Pectinatus* spp. are non-spore-forming motile rods with lateral flagella attached to the concave side of the cell

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body. They swim actively and appear X-shaped when cells are young and snake-like and longer when cells are older. They possess some features that are characteristic for Gram-positive bacteria (Haikara *et al.*, 1981) and are regarded as being intermediate between Gram-positive and -negative bacteria. Growth takes place between 15 and 40°C with an optimum around 32°C (Chelak and Ingledew, 1987), between pH 3.5 and 6 with an optimum at 4.5 (Chelak and Ingledew, 1987; Watier *et al.*, 1993) and in media containing up to 4.5% (w/v) of ethanol. During growth considerable amounts of propionic and acetic acids are produced as well as succinic and lactic acids and acetoin. *Pectinatus* spp. can also ferment lactic acid. Since lactic acid is the sole source of carbon in SMMP medium (see section 3.1), this medium is used for the selective isolation of *Pectinatus* spp. (and *Megasphaera* spp.) (Lee, 1994). The most characteristic feature of spoilage caused by *Pectinatus* spp. is extensive turbidity and an offensive 'rotten egg' smell brought by the combination of various fatty acids, hydrogen sulfide and methyl mercaptan (Lee *et al.*, 1978, 1980; Haikara *et al.*, 1981). This spoilage activity can cause serious damages for breweries.

### 2.2.2. *Megasphaera*

*Megasphaera* has emerged in breweries along with *Pectinatus* and is responsible for 3 to 7% of bacterial beer incidents (Back *et al.*, 1988; Back, 1994). They are non-spore-forming, nonmotile, mesophilic cocci that occur singly or in pairs and occasionally as short chains. This genus includes two species, *M. elsdeni* and *M. cerevisiae*. Since the first isolations in 1976 only *M. cerevisiae* has been blamed to be responsible for beer spoilage (Weiss *et al.*, 1979; Haikara and Lounatmaa, 1987; Lee, 1994). *M. cerevisiae* grows between 15 to 37°C with an optimum at 28°C and at pH values above 4.1. The growth is inhibited at ethanol concentrations above 2.8 (w/v) but is still possible up to 5.5 (w/v) (Haikara *et al.*, 1987; Lawrence, 1988). It is the most anaerobic species known to exist in the brewing environment (Seidel *et al.*, 1979). Beer spoilage caused by this organism results in a similar extreme turbidity as *Pectinatus* and the production of considerable quantities of butyric acid together with smaller amounts of acetic, isovaleric, valeric and caproic acids as well as acetoin (Seidel *et al.*, 1979). Like *Pectinatus*, the production of hydrogen sulfide causes a fecal odor in beer (Lee, 1994), which makes this bacterium one of the most feared organisms for brewers.

### 2.2.3. Other Gram-Negative Bacteria

In addition to the two genera described above, some other Gram-negative bacteria have been found to cause problems in the brewing industry. Anaerobic *Zymomonas* spp. have been found in primed beer to which sugar was added and in ale beer. *Zymomonas mobilis* is an aerotolerant anaerobe and grows above pH 3.4 and at ethanol concentrations below 10% (w/v) (van Vuuren, 1996). There is no

report of *Zymomonas mobilis* spoilage in lager beer, probably because of its selective fermentation character (non-fermentative of maltose, maltotriose but fermentative of glucose, fructose and sucrose). It produces high levels of acetaldehydes and hydrogen sulfide. Also another *Zymophilus* spp., *Z. raffinosivorans* has been reported as a beer spoiler (Schleifer *et al.*, 1990; Seidel-Rüfer, 1990). The genus *Zymophilus* is phylogenetically close to the genus *Pectinatus*. *Zymophilus* spp. can grow in beer, like *Pectinatus* spp., at pH values above 4.3-4.6 and ethanol concentrations below 5% (w/v). Also their beer spoilage activity is similar to that of *Pectinatus* spp. (Jespersen and Jakobsen, 1996).

Another Gram-negative bacterium *Selenomonas lacitifex* has also been reported to play a role in certain beer spoilage incidents but this species has hardly been studied. Historically a lot of attention of the brewing industry has been given to aerobic Gram-negative bacteria. Acetic acid bacteria i.e. *Gluconobacter* and *Acetobacter* used to be well-known to breweries. They convert ethanol into acetate, which results in vinegary off-flavor of beer. For reasons explained above such aerobes are no longer important in modern breweries.

*Hafnia protea*, formerly *Obesumbacterium proteus*, and *Rahnella aquatilis*, formerly *Enterobacter agglomerans*, have been detected in pitching yeasts but never in finished beer. They can retard the fermentation process. Beer produced with yeasts contaminated with *H. protea* has a parsnip-like or fruity odor and flavor (van Vuuren, 1996). Abnormally high levels of diacetyl and dimethyl sulfide were detected in beer produced from wort contaminated by *R. aquatilis* (van Vuuren, 1996).

Recently a novel strictly anaerobic Gram-negative bacterium was isolated from a brewery (Nakakita *et al.*, 1998). It is a rod-shaped bacterium with no flagella that can grow in beer at pH values above 4.3 and does not produce propionic acid. Genetic and phenotypical studies indicated that this bacterium is different from *Pectinatus*, *Zymomonas* and *Selenomonas* spp..

**Table 1. Beer Spoilage Bacteria**

<b>Gram-positive bacteria</b>	<b>Rod-shaped</b>	<b>Cocci</b>
	<i>Lactobacillus</i> spp.	<i>Pediococcus</i> spp.
	<i>Lb. brevis</i>	<i>P. damnosus</i>
	<i>Lb. brevisimilis</i>	<i>P. dextrinicus</i>
	<i>Lb. buchneri</i>	<i>P. inopinatus</i>
	<i>Lb. casei</i>	
	<i>Lb. coryneformis</i>	<i>Micrococcus</i> sp.
	<i>Lb. curvatus</i>	<i>M. kristinae</i>
	<i>Lb. lindneri</i>	
	<i>Lb. malefermentans</i>	
	<i>Lb. parabuchneri</i>	
	<i>Lb. plantarum</i>	
<b>Gram-negative bacteria</b>	<b>Rod-shaped</b>	<b>Cocci</b>
	<i>Pectinatus</i> spp.	<i>Megasphaera</i> sp.
	<i>P. cerevisiiphilus</i>	<i>M. cerevisiae</i>
	<i>P. frisingensis</i>	
	<i>P. sp.</i> DSM20764	
	<i>Selenomonas</i> sp.	<i>Zymomonas</i> sp.
	<i>S. lacticifex</i>	<i>Z. mobilis</i>
	<i>Zymophilus</i> sp.	
	<i>Z. raffinovorans</i>	

### 3. DETECTION OF BEER SPOILAGE BACTERIA

We have seen above that only a limited number of bacterial species are responsible for beer spoilage and that only a few species are major beer spoilage bacteria. For quality assurance of finished beer it is usually sufficient to control potential contaminations by *Lactobacillus brevis*, *Lb. lindneri*, *Pediococcus damnosus*, and *Pectinatus* spp.. Most studies on beer spoilage bacteria have focused on the taxonomical classification of these bacteria. These studies have made it possible to identify the bacteria detected in beer and breweries and to take the proper measures to control them.

#### 3.1. Culture Media

Along with the taxonomical studies a number of selective culture media for beer spoilage bacteria have been developed. European Brewery Convention recommends three media for the detection of lactobacilli and pediococci: MRS (de Man, Rogosa and Sharpe) agar supplemented with cycloheximide to prevent growth of aerobes such as yeasts and moulds, Raka-Ray medium supplemented with cycloheximide and VLB (Versuchs- und Lehranstalt für Brauerei in Berlin) S7-S. Other optional media are UBA (Universal Beer Agar) supplemented with cycloheximide, HLP (Hsu's *Lactobacillus* and *Pediococcus* medium), NBB (Nachweismedium für bierschädliche Bakterien), WLD (Wallerstein Differential), Nakagawa, SDA (Schwarz Differential Agar) and MRS modified by addition of maltose and yeast extract at pH 4.7. None of these media are suitable for detecting all strains of lactobacilli and pediococci but a combination of some of these media yields the best results. For the detection of *Pectinatus* and *Megasphaera* the following media are recommended: Concentrated MRS broth, PYF (Peptone, Yeast extract and Fructose) and Thioglycolate Medium for enrichment of beer, LL-Agar for growth in Lee tube, and UBA, NBB and Raka-Ray for routine analysis at breweries. For *Zymomonas* spp., *Zymomonas* Enrichment Medium is also recommended (EBC Analytica Microbiologica II, 1992). American Society of Brewing Chemists recommends UBA and Brewer's Tomato Juice Medium for general microbial detection and other media including LMDA (Lee's Multi-Differential Agar), Raka-Ray, BMB (Barney-Miller Brewery Medium) and MRS for the detection of lactic acid bacteria and SMMP (Selective Medium for *Megasphaera* and *Pectinatus*) (Methods of Analysis of the American Society of Brewing Chemists, Eighth Revised Edition, 1992). Brewery Convention of Japan also recommends the use of some of those media (BCOJ Biseibutu Bunsekihou, 1999). These culture media are listed in Table 2.

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**Table 2. Selective culture media for the detection of beer spoilage bacteria**

<b>Media</b>	<b>Bacteria</b>	<b>Recommended by<sup>3</sup></b>
MRS (de Man, Rogosa and Sharpe)	LAB <sup>1</sup>	EBC, ASBC, BCOJ
Raka-Ray	LAB, G(-) <sup>2</sup>	EBC, ASBC, BCOJ
VLB S7-S (Versuchs- und Lehranstalt für Brauerei in Berlin)	LAB	EBC, BCOJ
HLP (Hsu's <i>Lactobacillus</i> and <i>Pediococcus</i> medium)	LAB	EBC, BCOJ
WLD (Wallerstein Differential)	LAB	EBC, BCOJ
Nakagawa	LAB	EBC, BCOJ
SDA (Schwarz Differential Agar)	LAB	EBC, BCOJ
Concentrated MRS	G(-)	EBC, BCOJ
PYF (Peptone, Yeast extract and Fructose)	G(-)	EBC, BCOJ
Thioglycolate Medium	G(-)	EBC
LL-Agar	G(-)	EBC, BCOJ
UBA (Universal Beer Agar)	LAB, G(-)	EBC, ASBC, BCOJ
NBB (Nachweismedium für bierschädliche Bakteriën)	LAB, G(-)	EBC, BCOJ
Brewer's Tomato Juice Medium	LAB, G(-)	ASBC
LMDA (Lee's Multi-Differential Agar)	LAB	ASBC
BMB (Barney-Miller Brewery Medium)	LAB	ASBC
SMMP (Selective Medium for <i>Megasphaera</i> and <i>Pectinatus</i> )	G(-)	ASBC, BCOJ

<sup>1</sup> LAB, Lactic acid bacteria

<sup>2</sup> G(-), Gram-negative bacteria

<sup>3</sup> EBC, European Brewery Convention; ASBC, American Society of Brewing Chemists; BCOJ, Brewery Convention of Japan

The conventional detection method based on culturing of the organisms in these media has the significant disadvantage that is very time-consuming. One week or even longer is needed to obtain visible colonies on plates or turbidity in broths. Consequently, the products are often already released for sale before the microbiological results become available. Another problem is that these media are not species-specific. Media for the detection of beer spoiling lactic acid bacteria allow also growth of non-beer-spoilage species such as *Lactobacillus delbrueckii* and *Pediococcus acidilactici*. If the selectivity is increased by the addition of specific chemicals to these media, even longer detection times might be required.

### 3.2. Identification Methods

Following the bacterial detection in these media, species identification is needed. Besides the basic tests such as colony morphology, cell morphology, Gram-staining and catalase assays, also biochemical tests such as sugar fermentation pattern and chromatographic analysis of organic acids can be performed. Also specific detection and identification methods are used such as immunoassays with

polyclonal or monoclonal antibodies (Claussen *et al.*, 1975, 1981; Dolezil and Kirsop, 1976; Haikara, 1983; Gare, *et al.*, 1993; Sato *et al.*, 1994; Whiting *et al.*, 1992, 1999a, 1999b; Ziola *et al.*, 1999, 2000a, 2000b), DNA-DNA hybridization, DNA sequencing and PCR (Polymerase Chain Reaction) (Tsuchiya *et al.*, 1992, 1993, 1994; DiMichele and Lewis, 1993; Thompson *et al.*, 1994; Vogesor *et al.*, 1995a, 1995b; Yasui, 1995; Stewart and Dowhanick, 1996; Yasui *et al.*, 1997; Sakamoto, 1997; Sakamoto *et al.*, 1997; Satokari *et al.*, 1997, 1998; Juvonen and Satokari, 1999; Motoyama and Ogata, 2000; Bischoff *et al.*, 2001). These modern methods have been reviewed (Berney and Kot, 1992; Schmidt, 1992; Dowhanick and Russel, 1993; Dowhanick, 1995; Schofield, 1995; Hammond, 1996; Schmidt, 1999). Especially the application of PCR has recently significantly been improved (see Chapter 2).

### 3.3. Discrimination of Beer Spoilage Bacteria from Non-Spoilers

After detection and identification it is for some species necessary to identify the bacterium as an actual beer spoiler. While all strains belonging to *Pectinatus* spp. and *Megasphaera cerevisiae* have been reported to be capable of spoiling beer (Haikara, 1991), lactic acid bacteria include both beer spoilage and non-spoilage strains. Among *Lb. brevis* and *P. damnosus* most of the strains are capable of spoiling beer and only a few strains are not. On the other hand the number of beer spoilage strains in *Lb. casei*, *Lb. coryneformis* and *Lb. plantarum* is limited. Exceptionally, all strains of *Lb. lindneri* have been reported to be capable of spoiling beer (Rinck and Wackerbauer, 1987a, 1987b; Storgårds *et al.*, 1998).

Before the beginning of 1990s the only method was available for judging the beer spoiling potential of a bacterium, the so-called 'forcing test'. In this test the bacterium was re-inoculated into beer or beer enriched with concentrated nutrient medium. However, this test has proven to be far from practical for quality assurance since a few months are needed to obtain conclusive results.

More rapid procedures have been developed. The identification at the strain level can now be done at the genome level. Ribotyping, based on Southern hybridization with a ribosomal gene as a probe, has been successfully introduced (Motoyama *et al.*, 1998, 2000; Satokari *et al.*, 2000; Suihko and Haikara, 2000; Barney *et al.*, 2001). Fully automated ribotyping machines are now commercially available and only eight hours are needed to obtain conclusive results. AFLP (Amplified Fragment Length Polymorphisms) (Perpete *et al.*, 2001) and RAPD-PCR (Random Amplified Polymorphic DNA) (Savard *et al.*, 1994; Tompkins *et al.*, 1996) have also successfully been applied for bacterial strain identification as well as for identification of brewing yeasts. In these methods the genotype of each beer spoilage strain is registered in a database. A comparison of the genotype of a newly detected strain with registered genotypes will make a risk assessment possible.

Another approach is to determine the common physiological properties

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responsible for beer spoiling ability. For beer spoiling lactic acid bacteria the common physiological denominator is hop resistance, which allows growth of these bacteria in beer. However, measuring hop resistance by culturing in hop containing medium, is too time-consuming. It will be much faster to detect the physiological traits that cause hop resistance with immunoassays or PCR. Before this can be done the cause of the antibacterial activity of hop compounds and the mechanism(s) responsible for resistance towards hop compounds need to be known.

## 4. ANTIBACTERIAL ACTIVITY OF HOP COMPOUNDS

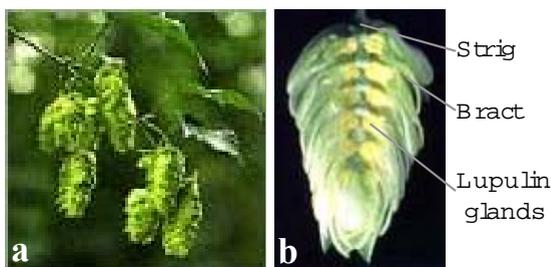
### 4.1. History of Hop Usage in Beer

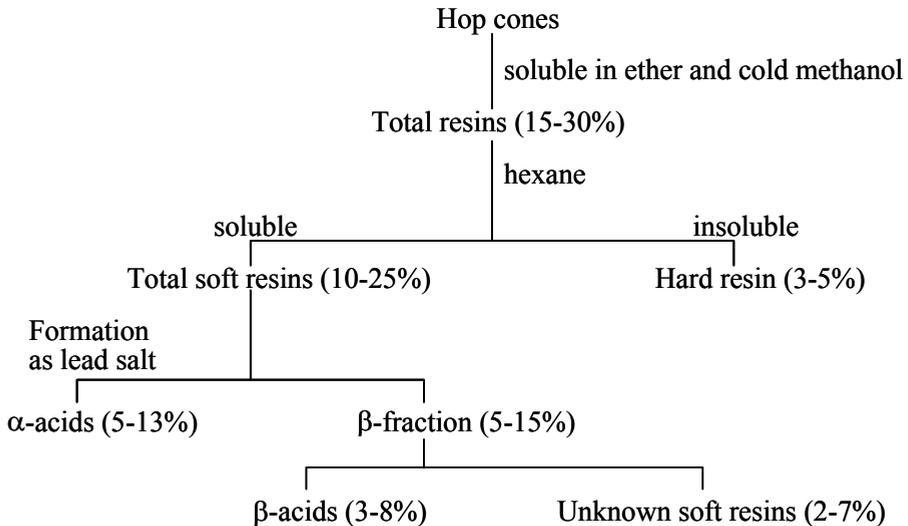
The comfortable bitterness experienced in beer drinking is characteristic for and is mainly caused by hop compounds. These hop compounds are present in the flowers of the hop plant, *Humulus lupulus*, L., which are added to the wort. This plant has been known for thousands of years. However, its use in beer is not as old as the history of beer itself (5,000 to 7,000 years). A few descriptions of hops as a beer additive as well as a decoration for gardens were found in documents of the sixth century B.C. German monks in the 12th century often used hops in beer making. In those days, as in ancient times, it was popular to use a variety of fruits, herbs and spices to flavor beer (so-called gruit beer). Initially the bitter taste from hops was not particularly appreciated. However, when in the 14th century beer production increased and beer was exported, the importance of hops in beer was gradually more and more appreciated, not only for its contribution to beer flavor but also for its contribution to the stability. Hopped beer can be preserved significantly longer than gruit beer. In 1516 Wilhelm IV, the lord of Bayern, enacted the ‘Reinheitsgebot (Purity Law)’ which ordered that beer must be made from barley, water and hops. Since then, the use of hops became more popular and standard. Many aspects of this law were adopted by other countries, which made hops indispensable for the brewing industry.

### 4.2. Hop Plant

The hop plant is a vine, belonging to the family of hemp (Fig. 1). It is dioecious and blooms yearly. Nowadays it is mainly cultured for the brewing industry. Only the female flowers, the so-called cones, are used for beer. The mature cones contain golden resinous granules, the lupulin, which are the most important part of the flower for the bitterness and preservation of beer. Hop resins are extracted and fractionated as shown in Fig. 2.

**Figure 1. Hop plant. (a)** Hop cones at the end of the vine. **(b)** A vertical section of hop cone. Lupuline glands locate on the base of each bract. They contain the hop resins and essential oils which give beer a unique flavor.



**Figure 2. Fractionation of hop resins** (Hough *et al.*, 1971)

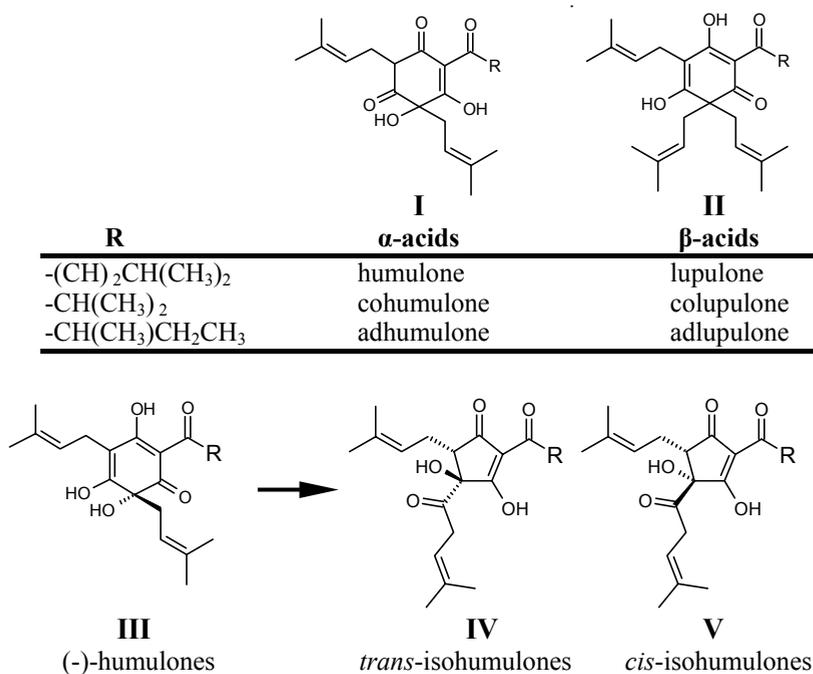
### 4.3. Antibacterial Compounds in Hops

Hop chemistry has been developed since 19th century and has been extensively reviewed (Verzele, 1986; Moir, 2000). Research has especially been focused on the antibacterial properties of hop compounds and the bitter substances derived from hops. This research goes back to 1888 when Hayduck showed first that antiseptic properties of the hops are due to the soft resins (Hayduck, 1888). In the Institute of Brewing of the United Kingdom, Walker conducted from 1922 till 1941 a long-term investigation on ‘the preservative principles of hops’ (Pyman *et al.*, 1922; Walker, 1923a, 1923b, 1924a, 1924b, 1925, 1938, 1941; Hastings *et al.*, 1926; Hastings and Walker, 1928a, 1928b, 1929; Walker and Hastings, 1931, 1933a, 1933b; Walker *et al.*, 1931, 1932, 1935, 1940; Walker and Parker, 1936, 1937a, 1938, 1940a, 1940b). The study focused on the antiseptic properties of  $\alpha$ -acid fraction (humulone) and  $\beta$ -acid fraction (lupulone).

The  $\alpha$ -acid fraction is a mixture of homologous compounds, the  $\alpha$ -acids, which are not transferred as such to beer. During the wort boiling stage in the brewing process,  $\alpha$ -acids are converted by a rearrangement or isomerization to iso- $\alpha$ -acids, which are much more soluble and bitterer than the original compounds. This conversion, which is very important in hop chemistry, was advanced first in 1888 (Hayduck). Wieland *et al.* (1925) suggested that the hydrolysis of humulone to humulinic acid proceeded via an intermediate. Windisch *et al.* (1927) investigated the humulone boiling products under alkaline conditions and isolated a resinous

and bitter oil termed “Resin A” with chemical properties similar to the intermediate and isomeric with humulone. Around 1950, Rigby and Bethune showed that  $\alpha$ -acid fraction is a mixture of three major compounds; humulone, cohumulone and adhumulone (Figure 3) (Rigby and Bethune, 1952, 1953). The bittering compounds of beer were found to comprise three major analogues of these three  $\alpha$ -acids, which are now known as iso- $\alpha$ -acids; isohumulone, isocohumulone and isoadhumulone (Figure 3). Stereoisomers (*cis*- and *trans*-) exist for each iso- $\alpha$ -acid. Finally the chemical structure and configuration of naturally occurring (-)-humulone (De Keukeleire and Verzele, 1970) and isohumulones (De Keukeleire and Verzele, 1971) were elucidated. The isomerization yield of  $\alpha$ -acids during wort boiling process is low [typically of the order of 30% (Hughes, 2000)] due to relatively acidic condition of wort (ca. pH 5.2) and the adsorption to the wort coagulum during boiling and fermentation.

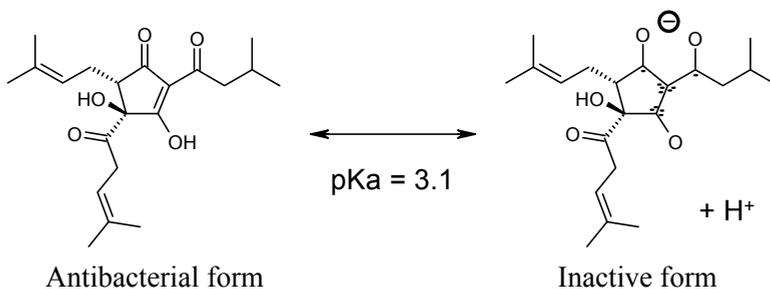
$\beta$ -acids or lupulones in hops are very poorly soluble in wort and beer and cannot undergo the same isomerization processes as  $\alpha$ -acids. Consequently, they are not transferred to beer and have no direct value in brewing.



**Figure 3. Chemical structures of hop compounds.** The name of each  $\alpha$ -acid (I) and  $\beta$ -acid (II) is dependent on its side chain. During wort boiling process,  $\alpha$ -acids (naturally *R*-body; III) are isomerized to result in stereoisomers of *trans*-isohumulones (IV) and *cis*-isohumulones (V).

#### 4.4. Antibacterial Mechanism of Hop Compounds

The antibacterial activities of  $\alpha$ -acid (humulone) and  $\beta$ -acid (lupulone) have been studied before 1950. Their antibacterial activities are higher than of iso- $\alpha$ -acids but they dissolve to less extent in beer and water. Studies of the antiseptic properties of hopped wort and hop boiling product showed that they inhibit growth of Gram-positive bacteria but not of Gram-negative bacteria (Shimwell, 1937a; Walker and Blakebrough, 1952). It was first reported by Shimwell (1937a) that the antiseptic potency of hop increases at lower pH. Interestingly, he predicted that the antiseptic potency of hop is associated with permeability changes of the bacterial cell wall (1937b). The 'bacteriostatic power' was also studied of hop compounds, including humulone and the humulone boiling product (Walker and Blakebrough, 1952). The humulone boiling product had less bacteriostatic potency in malt extract (pH 5.5) and wort (pH 5.2) than the original humulone, while its potency was the same at pH 4.3, the pH of beer. The hop constituents (lupulone, humulone, isohumulone and humulinic acid) were found to cause leakage of the cytoplasmic membrane of *Bacillus subtilis*, resulting in the inhibition of active transport of sugar and amino acids (Teuber and Schmalreck, 1973). Subsequently inhibition of respiration and synthesis of protein, RNA and DNA was also observed. Since the iso- $\alpha$ -acids are mainly present in beer among the hop resins and their derivatives, a precise investigation of the antibacterial activity of iso- $\alpha$ -acids was needed for understanding the preservation or bacterial stability of beer. The molecular mechanism of antibacterial activity of iso- $\alpha$ -acids and the effects of pH of the growth medium and other variables on the antibacterial activity of hop compounds were investigated after 1990 (Simpson and Smith, 1992). Hop compounds are weak acids and the undissociated forms are mainly responsible for inhibition of bacterial growth (Fig. 4).



**Figure 4. Dissociation of *trans*-isohumulone.** (Fernandez and Simpson, 1995b)

In *Lb. brevis* (Simpson, 1993b) *trans*-isohumulone reduces the uptake of leucine and causes slow leakage of accumulated leucine. *trans*-Isohumulone dissipates

effectively the transmembrane pH gradient ( $\Delta\text{pH}$ ) of the proton motive force but not the transmembrane electrical potential ( $\Delta\psi$ ). Inhibition of  $\text{H}^+$ -ATPase activity was not observed. Potentiometric studies revealed that undissociated *trans*-isohumulone acts most likely as an ionophore, catalyzing electroneutral influx of undissociated isohumulone, internal dissociation of ( $\text{H}^+$ )-isohumulone and efflux of the complex of isohumulone with divalent cations such as  $\text{Mn}^{2+}$ . This cation is known to be present at high concentrations in lactobacillus cells (Archibald and Fridovich, 1981a, 1981b; Archibald and Duong, 1984). The result of this activity is a decrease of the pH gradient across the membrane. It was reported that the antibacterial activity of *trans*-isohumulone can be influenced by the presence of cations in the medium. Protonophoric activity of *trans*-isohumulone requires the presence of monovalent cations such as  $\text{K}^+$ ,  $\text{Na}^+$  or  $\text{Rb}^+$  and increases with the concentration of these monovalent cations (Simpson and Smith, 1992). *trans*-Isohumulone cannot bind  $\text{K}^+$  unless a divalent cation, such as  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Ca}^{2+}$  or a trivalent cation, such as  $\text{Li}^{3+}$  and  $\text{Al}^{3+}$ , is present in the medium (Simpson *et al.*, 1993; Simpson and Hughes, 1993). Thus, the ability of hop compounds to bind simultaneously two or more cations may be crucial for their antibacterial action but the reason has been still unclear.

The properties of other hop acids are similar to those of *trans*-isohumulone and it is likely that the mechanism of their antibacterial activities is also similar. Some strains of lactic acid bacteria, which are sensitive to *trans*-isohumulone, are also sensitive to (-)-humulone and colupulone and other strains resistant to *trans*-isohumulone are also resistant to the related compounds (Fernandez and Simpson, 1993). The antibacterial activities of 6 naturally occurring iso- $\alpha$ -acids, 5 chemically reduced iso- $\alpha$ -acids and a reduced iso- $\alpha$ -acids mixture were higher at lower pH-values while more hydrophobic reduced iso- $\alpha$ -acids were found to be far more antibacterial than their naturally occurring analogues (Price and Stapely, 2001).

### 5. HOP RESISTANCE IN LACTIC ACID BACTERIA

Beer spoiling lactic acid bacteria have to be hop resistant in order to grow in beer. The understanding and elucidation of the mechanism of hop resistance is not only of scientific interest but is also important for the microbial control in brewing industry to predict the beer spoiling ability of lactic acid bacteria.

#### 5.1. Variation of Hop Resistance

The extent of hop resistance varies between bacteria. Among beer spoiling lactic acid bacteria *Lb. brevis* is so far the most resistant to hop compounds. The degree of hop resistance varies among the different strains of *Lb. brevis* (Hough *et al.*, 1957; Harris and Watson, 1960; Simpson and Fernandez, 1992; Fernandez and Simpson, 1993). Hop resistance of lactobacilli decreases upon prolonged serial subculturing in the absence of hop compounds (Shimwell, 1936c; Yamamoto, 1958; Richards and Macrae, 1964). Hop resistance was thought to be caused by immunity acquired by prolonged contact with hop compounds under brewing conditions (Shimwell, 1937a). The necessity of beer spoiling lactic acid bacteria to acclimatize to beer or hop compounds in order to reproduce in beer (Yamamoto, 1958) was solidly documented by Richards and Macrae in 1964. Hop resistance increased 8 to 20 fold in strains of lactobacilli upon serial subculturing in media containing increasing concentrations of hop compounds, while subculturing of resistant populations in the absence of hop compounds resulted gradually in decreased hop resistance. It took about one year of subculturing in unhopped beer to maximally reduce hop resistance of lactobacilli, indicating that the acquired hop resistance can be a very stable property (Shimwell, 1936c). However, organisms isolated from spoiled beer frequently fail to grow upon reinoculation in beer. Preculturing in the presence of sub-inhibitory concentrations of isohumulone is needed in order to make growth in beer possible (Simpson and Fernandez, 1992). The stability of hop resistance in *Lb. brevis* appears to vary from strain to strain. The hop resistance in *Lb. brevis* strain BSO310 could not be altered by plasmid curing or mutation induced with ultraviolet light (Fernandez and Simpson, 1993), suggesting that it may be generally a stable character, both phenotypically and genetically. The *Lb. brevis* strain ABBC45 can develop hop resistance in the same way as observed by Richards and Macrae (Sami *et al.*, 1997a). Hop resistance increased with the copy number of plasmid pRH45. When *Lb. brevis* ABBC45 was cured from this plasmid by serial subculturing in the absence of hop compounds, the degree of hop resistance decreased (Sami *et al.*, 1998; Suzuki *et al.*, 2002). This plasmid contains the *horA* gene that codes for a polypeptide that is 53% identical to LmrA, the lactococcal ATP-binding cassette (ABC) multidrug transporter (van Veen *et al.*, 1996). HorA protein was expressed heterologously in *Lactococcus lactis* and found to function as an ABC-type multidrug transporter and to excrete hop compounds (Sakamoto *et al.*, 2001).

## 5.2. Features of Hop Resistance

The pattern of resistance or sensitivity of several species of lactobacilli to isohumulone is similar to that of the related hop acids humulone and lupulone (Richards and Macrea, 1964; Fernandez and Simpson, 1993). Fernandez and Simpson (1993) compared the properties of hop resistant strains of lactobacilli and pediococci with those of sensitive strains. No obvious correlation was found between hop resistance and cell morphology, colony morphology, pH range for growth, sugar utilization profile, products of metabolism, manganese requirement and sensitivity to superoxide radicals, expression of cellular proteins, and resistance to various antibacterial agents. However, differences were found in the transmembrane pH gradient ( $\Delta\text{pH}$ ) and the cellular ATP pool. Because hop compounds act as protonophores that dissipate the  $\Delta\text{pH}$  across the cellular membrane (Simpson, 1993a, 1993b), these differences are of great importance for understanding the mechanism of hop resistance.

## 5.3. Mechanisms of Hop Resistance

The molecular structure of antibacterial agents might supply an insight in the mechanism of resistance. Resistance to *trans*-isohumulone also results in resistance to (-)-humulone and colupulone (Fernandez and Simpson, 1993), suggesting a common mechanism of resistance against a broad range of hop acids. *trans*-Isohumulone and (-)-humulone have three hydrophobic side chains while colupulone has four. The side chains are attached to a five-membered ring of *trans*-isohumulone, but to a six-membered ring of (-)-humulone and colupulone (Fig. 3). On the other hand resistance was not observed to other ionophores (nigericin, A23187, CCCP, monesin), weak acid food preservatives (sorbic acid, benzoic acid), solvents (ethanol), or antibiotics (ampicillin, cefamandole, vancomycin) (Fernandez and Simpson, 1993). The resistance mechanism might be specific for the  $\beta$ -triketone group of the hop acids, which plays an essential role in the antibacterial action (Simpson, 1991).

Micro-organisms have developed various ways to resist the toxicity of antibacterial agents:

(i) enzymatic drug inactivation. A well known example is  $\beta$ -lactamase which hydrolyzes the  $\beta$ -lactam ring into innocuous substrates. In hop resistant strains of *Lb. brevis*, neither conversion nor inactivation of *trans*-isohumulone was found (Simpson and Fernandez, 1994).

(ii) target alteration. Cellular targets can be altered by mutation or enzymatic modification in such a way that the affinity of the target for the antibiotics is reduced. In the case of *trans*-isohumulone, the target site is the cell membrane (Teuber and Schmalreck, 1973; Schmalreck *et al.*, 1975; Simpson, 1993a, 1993b). A 'sake (Japanese rice wine)' spoilage bacterium, *Lb. heterohiochii*, contains extremely long chain fatty acids in its membrane (Uchida, 1974), which may play a

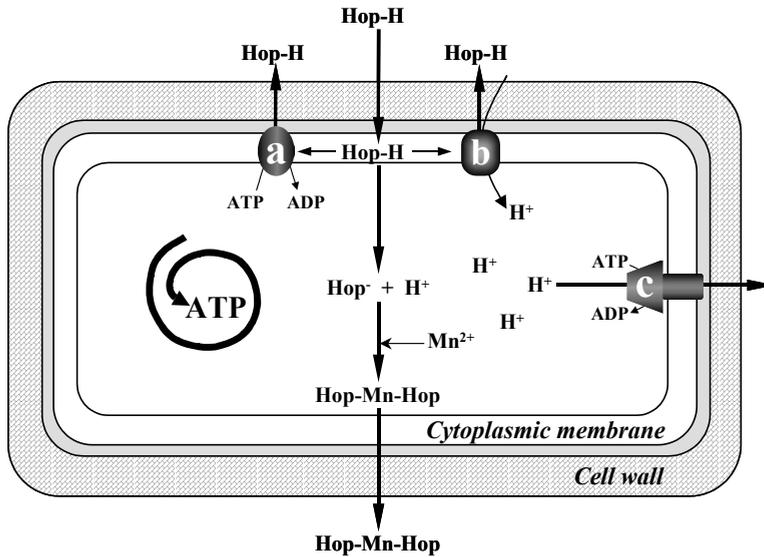
## CHAPTER 1

role in ethanol resistance (Ingram, 1984). It is possible but not yet investigated that hop resistant *Lb. brevis* has also a changed lipid composition of its membrane to lower the permeability to hop compounds.

(iii) inhibition of drug influx. The outer membrane of Gram-negative bacteria restricts the permeation of lipophilic drugs, while the cell wall of the Gram-positive mycobacteria has been found to be an exceptionally efficient barrier. The permeation of hop compounds might also be affected by the presence of a galactosylated glycerol teichoic acid in beer spoilage lactic acid bacteria (Yasui and Yoda, 1997b).

(iv) active extrusion of drugs. The presence of (multi)drug resistance pump in the cytoplasmic membrane of many bacteria has been extensively documented (Putman *et al.*, 2000a). A multidrug resistance pump HorA (Sakamoto *et al.*, 2001) has been found in *Lb. brevis* ABBC45, which is overexpressed when exposed to hop compounds (Sami, 1999). In addition a proton motive force dependent hop excretion transporter was suggested in this strain (Suzuki *et al.*, 2002).

(v) other mechanisms to tolerate the toxic effects of drugs. Since hop compounds act as protonophores and dissipate the transmembrane pH gradient ( $\Delta\text{pH}$ ), the cells could respond by increasing the rate at which protons are expelled. The hop resistant strains maintain a larger  $\Delta\text{pH}$  than hop sensitive strains (Simpson and Fernandez, 1993) and *Lb. brevis* ABBC45 increases its  $\text{H}^+$ -ATPase activity upon acclimatization to hop compounds (Sakamoto *et al.*, 2002). The ATP pool in hop resistant strains was also found to be larger than in hop sensitive strains (Simpson and Fernandez, 1993; Okazaki *et al.*, 1997). The ability of hop resistant strains to produce large amounts of ATP in the cell is needed for the increased activity of the  $\text{H}^+$ -ATPase and for the hop extruding activity of HorA. It is interesting that these responses occurred also in hop sensitive strains at sub-inhibitory concentration of *trans*-isohumulone (Simpson, 1993a; Simpson and Fernandez, 1993). However, at higher concentrations both the  $\Delta\text{pH}$  and the ATP pool decreased in the hop sensitive strains, but not in the hop resistant strains. A role of HitA in hop resistance was suggested (Hayashi *et al.*, 2001). It is about 30% identical to the natural resistance-associated macrophage proteins (Nramp), which function as divalent-cation transporters in many prokaryotic and eukaryotic organisms. HitA could play a role in transport of divalent cations while isohumulone has been claimed to exchange protons for cellular divalent cations such as  $\text{Mn}^{2+}$ . Thus a simple mechanism of hop resistance does not appear to exist. The resistance mechanisms found so far in *Lb. brevis* are illustrated in Fig. 5.



**Figure 5. Mechanisms of hop resistance.** Resistance to hop compounds is conferred by a number of processes. Hop compounds (Hop-H) are expelled from the cytoplasmic membrane in hop resistant cells by HorA (**a**) (Sakamoto *et al.*, 2001) and probably also by a pmf-dependent transporter (**b**) (Suzuki *et al.*, 2002). When hop compounds enter the cytoplasm they dissociate due to the higher internal pH into hop anions and protons. Overexpression of H<sup>+</sup>-ATPase (**c**) results in increased proton pumping and pmf generation (Sakamoto *et al.*, 2002). Hop anions can trap divalent cations such as Mn<sup>2+</sup> and diffuse out of the cell. The hop resistant strains can generate more ATP than hop sensitive strains (whirlpool).

### 6. BEER SPOILING ABILITY IN LACTIC ACID BACTERIA

Hop resistance is crucial to beer spoiling ability of lactic acid bacteria. In many bacteria hop resistance mechanisms need to be induced before growth in beer is possible. For this induction some bacteria need to be exposed to sub-inhibitory concentrations of hop compounds (Simpson, 1993).

#### 6.1. Factors Affecting the Growth in Beer

Beer spoilage and bacterial growth depend on the strain and the type of beer. The ability of 14 hop-resistant lactic acid bacterial strains, including *Lb. brevis* and *P. damnosus* strains, was investigated for their capacity to grow in 17 different lager beers with the biological challenge test (Fernandez and Simpson, 1995a). A statistical analysis of the relationship between spoilage potential and 56 parameters of beer composition revealed a correlation with eight parameters: pH, beer color, the content of free amino nitrogen, total soluble nitrogen content and the concentrations of a range of individual amino acids, maltotriose, undissociated SO<sub>2</sub> and hop compounds. The effects of dissolved carbon dioxide (CO<sub>2</sub>) and phenolic compounds including catechin, gallic, phytic and ferulic acids on beer spoilage were also investigated (Hammond *et al.*, 1999). CO<sub>2</sub> was found to inhibit the growth of lactobacilli at the concentrations present in typical beer but stimulate at the lower concentrations. Among the phenolic compounds, ferulic acid, a component of barley cell wall and hence present in all beers, exerted a stronger antibacterial activity after enzymatic conversion into 4-vinyl guaiacol. Organic acids in beer may also influence bacterial growth but this aspect has hardly been studied.

#### 6.2. Prediction of Beer Spoilage by Lactic Acid Bacteria

A number of attempts have been made to develop methods to predict beer spoiling ability. For lactic acid bacteria hop resistance is the key factor. As described above, some factors have been identified to cause hop resistance. Rapid procedures for detecting these factors would be very beneficial for microbial control in breweries. A set of PCR primers have been made that can specifically detect the *horA* gene or its homologues in a wide range of lactobacilli (Sami *et al.*, 1997b). Most *horA* positive strains were found to have beer spoiling ability, indicating that this is a very useful prediction method. Another prediction method is based on ATP pool measurements in lactobacillus cells (Okazaki *et al.*, 1997).

Polyclonal and monoclonal antibodies specific only for beer spoilage strains have been reported. A series of antisera were made against the *Lactobacillus* group E antigen, a cell wall glycerol teichoic acid beneath the S-layer protein (Yasui *et al.*, 1992, 1995; Yasui and Yoda, 1997a) and known to be present in *Lb. brevis*, *Lb. buchneri*, *Lb. delbrueckii* subsp. *lactis* and subsp. *bulgaricus* (Sharpe, 1955; Sharpe *et al.*, 1964). When the beer spoilage strain *Lb. brevis* 578 was used as an antigen,

the resulting antiserum reacted specifically with other beer spoilage strains of *Lb. brevis* although they had been cultured in modified NBB medium (Nishikawa *et al.*, 1985) which does not contain any hop compounds or beer. Surprisingly, this antiserum also reacted with beer spoilage strains of *P. damnosus*, but not with any strains of *Lb. lindneri* (Yasui, and Yoda, 1997a). Galactosylated glycerol teichoic acid was found to be the most likely epitope that presumably selectively increases the cell barrier to hop compounds (Yasui and Yoda, 1997b). Three monoclonal antibodies specific for beer spoilage ability of lactic acid bacteria were obtained by immunizing mice with cells cultured in beer (Tsuchiya *et al.*, 2000). The monoclonal antibody raised against *Lb. brevis* reacted with all beer spoilage strains of *Lb. brevis* and several beer spoilage strains of *P. damnosus*, but not with non-spoilage strains of *Lb. brevis*, *P. damnosus* and other lactic acid bacteria. The monoclonal antibody raised against *P. damnosus* reacted significantly with all beer spoilage strains of *P. damnosus* and weakly with many of the beer spoilage strains of *Lb. brevis*. On the other hand the monoclonal antibody raised against *Lb. lindneri* reacted specifically only with *Lb. lindneri*. The reactivity of the still unknown antigens did not change regardless of the presence of hop compounds in their culture media.

D-lactate dehydrogenase (LDH) of 60 strains of *Lb. brevis*, including 44 beer spoiling strains and 16 non-spoiling strains, was also investigated. The strains could be divided in five groups (A, B, C, D and E) on the basis of the mobility of their D-LDH in native polyacrylamide gels (Takahashi *et al.*, 1999). Forty out of 44 beer spoilage strains were classified to the group B suggesting a relationship between the D-LDH profile and beer spoiling ability. The purified D-LDH of those groups had different pH and temperature optima and isoelectric points. Especially the temperature optimum of 50°C of the Group B D-LDH is significantly lower than that of the other D-LDHs (60°C).

Except for the forcing test, none of the other methods so far available can predict beer spoiling ability of all strains. Since hop resistance in lactic acid bacteria does not appear to be a general feature of all beer spoiling bacteria, combination of several methods will be required to detect all potential beer spoiling strains.

