Physiological implications of class IIa bacteriocin resistance in *Listeria monocytogenes* strains

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High-level resistance to class IIa bacteriocins has been directly associated with the absence of EIIAB\textsuperscript{Man} (MptA) subunit of the mannose-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS) (EII\textsubscript{Man}) in *Listeria monocytogenes* strains. Class IIa bacteriocin-resistant strains used in this study were a spontaneous resistant, *L. monocytogenes* B73-MR1, and a defined mutant, *L. monocytogenes* EGDe-mptA. Both strains were previously reported to have the EIIAB\textsuperscript{Man} PTS component missing. This study shows that these class IIa bacteriocin-resistant strains have significantly decreased specific growth and glucose consumption rates, but they also have a significantly higher growth yield than their corresponding wild-type strains, *L. monocytogenes* B73 and *L. monocytogenes* EGDe, respectively. In the presence of glucose, the strains showed a shift from a predominantly lactic-acid to a mixed-acid fermentation. It is here proposed that elimination of the EIIAB\textsuperscript{Man} in the resistant strains has caused a reduced glucose consumption rate and a reduced specific growth rate. The lower glucose consumption rate can be correlated to a shift in metabolism to a more efficient pathway with respect to ATP production per glucose, leading to a higher biomass yield. Thus, the cost involved in obtaining bacteriocin resistance, i.e. losing substrate transport capacity leading to a lower growth rate, is compensated for by a higher biomass yield.

INTRODUCTION

Food-associated strains of lactic acid bacteria frequently produce antimicrobial compounds referred to as class IIa bacteriocins (Ennahar et al., 2000a). Class IIa bacteriocins have been grouped based on their high homology, conserved N-terminal YGNGV motif and effective antilisterial activity (Klaenhammer, 1993; Ennahar et al., 2000b; Héchard & Sahl, 2002). The potential application of class IIa bacteriocins as food preservatives has been extensively studied in the search for safe, non-toxic, antimicrobial food additives. However, the frequent occurrence of resistance has become an increasingly important concern, since it reduces the value of adding class IIa bacteriocins to foods (Gravesen et al., 2002a; Ennahar et al., 2000b).

Investigations into the mechanism of class IIa bacteriocin resistance shows strong evidence for one prevalent mechanism among various listerial strains and *Enterococcus faecalis* (Gravesen et al., 2002b; Héchard et al., 2001). This mechanism involves the absence of the EIIAB subunit of a mannose-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS) (Gravesen et al., 2002b). PTS is a group translocation sugar transport system where the sugar concomitant with transport is phosphorylated. The phosphate group is transferred via a number of enzymes from phosphoenolpyruvate to the sugar (Lengele et al., 1994; Postma et al., 1993; Tchieu et al., 2001; Siebold et al., 2001). An insertional inactivation of the mptA gene encoding the EIIAB subunit that resides on the tricistronic mptACD operon of EII\textsuperscript{Man} in *L. monocytogenes* resulted in a high level of resistance to class IIa bacteriocins (Dalet et al., 2001; Gravesen et al., 2002b). It has been suggested that the EII\textsuperscript{Man} PTS membrane component or permease (MptD) could play a role as a possible target for class IIa bacteriocins (Dalet et al., 2001; Héchard & Sahl, 2002; Gravesen et al., 2002a).

Carbohydrates are required by *L. monocytogenes* as the primary free-energy source for growth, with glucose being the preferred source (Pine et al., 1989; Premaratne et al., 1991). There is evidence for the presence of two glucose transport systems in *L. monocytogenes*, a high-affinity PTS and a low-affinity proton-motive-force-driven system (Parker & Hutkins, 1997). There are indications that the mannose PTS transports glucose in *L. monocytogenes* (Dalet et al., 2001) and this PTS is also known to transport mannose and 2-deoxyglucose (Chaillou et al., 2001; Romick et al., 1996). In many lactic acid bacteria and streptococci,

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**Abbreviation:** PTS, phosphoenolpyruvate-dependent phosphotransferase system.
transport and phosphorylation of glucose occurs mainly
via a mannose PTS (Chaillou et al., 2001; Vadeboncoeur &
Pelletier, 1997), which may be similar for L. monocytogenes.

The aim of this study was to investigate the effect of the
missing MptA subunit of the EI1Man on glucose metabolism
in class IIA bacteriocin-resistant L. monocytogenes strains.
We focused on glucose consumption rates and analysis
of the end products of glucose metabolism. The growth
patterns of L. monocytogenes were also analysed in this
study in brain–heart infusion (BHI) culture medium with or
without added glucose as a free-energy source. All studies
were done on two wild-type, sensitive L. monocytogenes
strains, and their corresponding class IIA-resistant variant,
of which one was a spontaneous mutant and the other a
genetically defined mutant.

METHODS

Bacterial strains and growth conditions. All strains were grown
in glucose-free BHI broth (Difco), supplemented, or not supple-
mented, with 10 mM glucose (Associated Chemical Enterprises,
Glenvista, South Africa), according to the requirements of the study.
The strains were cultivated at 37°C without shaking, in tightly
capped Spectronic tubes or in Schott bottles. The L. monocytogenes
strains used were the following: wild-type food isolate L. monocytogenes
B73, and corresponding class IIA bacteriocin spontaneous
mutant L. monocytogenes B73-MR1; wild-type clinical isolate L.
monocytogenes EGDe; and corresponding insertionally inactivated
mptA mutant L. monocytogenes EGDe-MR1 (Gravesen et al.,
2002b; Hechard et al., 2001), referred to as L. monocytogenes EGDe-mptA,
which displays resistance to class IIA bacteriocins. Media used to
grow L. monocytogenes EGDe-mptA were supplemented with 5 μg
erthyromycin ml⁻¹.

Growth analysis. Bacterial growth was monitored using optical
density (OD) at 600 nm. Dry weight measurements were calibrated
against OD₆₀₀ measurements. An OD₆₀₀ value of 1·0 corresponds
to 0·64 g dry weight l⁻¹. Specific growth rates were calculated from
the growth absorbance data collected from Spectronic tube cultures,
and the same cultures were sampled for analysis of end products
of fermentation. In a separate experiment, samples were taken from
Schott bottle cultures, at regular intervals from early-exponential
phase through to stationary phase, for monitoring of glucose
consumption.

Quantification of glucose and fermentation end products. Samples collected for HPLC analysis were prepared and analysed
as described in Ward et al. (2000). Samples were analysed for
glucose, lactate, pyruvate, acetate, formate and ethanol. Glucose
was also determined enzymically using a linked hexokinase/glucose-
6-phosphate dehydrogenase assay. The buffer for the assay contained
890 mM Tris/HCl buffer pH 7·6, 2·38 U hexokinase, 1·19 U glucose-
6-phosphate dehydrogenase, 1·26 mM ATP, 1·27 mM NADP and
10 mM MgSO₄. Product analysis allowed the calculation of carbon
recovery, glucose yields, ATP yields and glucose consumption rates.

Calculations and statistical analysis. Calculations of specific
growth rates and Student’s t-tests were done using GRAPHPAD PRISM
3·0 (GRAPHPAD software, San Diego, CA, USA) and MATHEMATICA
(Wolfram Research; http://www.wolfram.com).

RESULTS

Growth in the presence or absence of glucose

To investigate the physiological implications of acquiring
class IIA bacteriocin resistance, growth was followed in
wild-type and class IIA bacteriocin-resistant strains grown
on BHI in the absence or presence of glucose (Fig. 1). Comparing the specific growth rate of the wild-type strains
and the respective resistant strains, we see that in the
presence of glucose the wild-type strains have a higher
growth rate, while in the absence of glucose the resistant
strains grow faster (Table 1). Biomass concentrations of
resistant strains and their respective wild-type strains were
comparable in the absence of glucose, while in the presence
of glucose the resistant strains resulted in a higher final
biomass than the respective wild-type strains (Table 1). Biomass yield on glucose, calculated as the difference in
biomass concentration in the presence and absence of
glucose divided by the glucose used, indicated higher values
for the resistant strains (respectively, 29·1 and 45·1 g dry

![Fig. 1](image-url). Growth of L. monocytogenes strains in BHI supplemented with 10 mM glucose (a) and BHI without glucose (b).
Growth studies were carried out at least in duplicate for all the strains. ■, Wild-type B73; ▲, class IIA-resistant mutant
B73-MR1; ▼, wild-type EGDe; ●, class IIA-resistant, insertionally inactivated mptA mutant EGDe-mptA.
Table 1. Maximum specific growth rate and biomass of wild-type and class IIA bacteriocin-resistant *L. monocytogenes* strains in BHI broth supplemented, or not supplemented, with glucose

Experimental values represent a mean of at least two independent measurements, and standard deviations are shown in parentheses.

<table>
<thead>
<tr>
<th><em>L. monocytogenes</em> strain</th>
<th>Specific growth rate (h^{-1})</th>
<th>Biomass (OD_{600})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BHI with 10 mM glucose</td>
<td>BHI without glucose</td>
</tr>
<tr>
<td>B73</td>
<td>0.68 (0.018)</td>
<td>0.70 (0.014)</td>
</tr>
<tr>
<td>B73-MR1*</td>
<td>0.46 (0.008)†</td>
<td>0.83 (0.007)†</td>
</tr>
<tr>
<td>EGDe</td>
<td>0.84 (0.028)</td>
<td>0.52 (0.027)</td>
</tr>
<tr>
<td>EGDe-mptA*</td>
<td>0.70 (0.015)†</td>
<td>0.67 (0.011)</td>
</tr>
</tbody>
</table>

*Indicates the class IIA-resistant *L. monocytogenes* strains.
†Represents a significantly different (P<0.05) growth rate or growth yield (biomass) of the resistant strain compared to the corresponding wild-type strain.

Fermentation analysis

The difference in biomass yields in the different cultures was further investigated by analysing the fermentation products. BHI is a rich medium and, even without an additional free-energy source added to the medium, significant growth was observed. However, the final biomass concentrations were much lower as compared to the cultures to which 10 mM of glucose was added. Glucose consumed by the bacteria was completely converted to fermentation products, indicating that it served as free-energy source (not as carbon source) and that biomass formation in BHI was limited by the availability of a free-energy source. Only very low concentrations of fermentation products were formed during growth on BHI (without glucose), typically 3 mM of formate and 2 mM of acetate (data not shown). Lactate, acetate, ethanol and formate were typical fermentation products observed when the bacterial strains were grown in the presence of glucose (Table 2). A marked change in fermentation pattern was observed between the wild-type strains and the bacteriocin-resistant strains. The wild-type strains showed a homolactic type of fermentation (i.e. 83 and 94 % of all product carbon was present in lactate for B73 and EGDe, respectively), while the fermentation pattern in the resistant strains was shifted more towards a mixed-acid type of fermentation (47 and 50 % of all product carbon was present in lactate for, respectively, B73-MR1 and EGDe-mptA). Since a mixed-acid fermentation has a higher ATP per glucose yield as compared to homolactic fermentation, the change in metabolism in the resistant strains is at least qualitatively in agreement with the observed higher biomass yields in these strains.

Glucose consumption rates

Realizing that the EIIAB\textsuperscript{Man} is used for glucose transport in *L. monocytogenes*, we investigated whether the reduced

Table 2. Fermentation product analysis and carbon recovery of *L. monocytogenes* strains grown in BHI supplemented with 10 mM glucose

The concentration values represent a mean of at least three independent measurements, and standard deviations are shown in parentheses.

<table>
<thead>
<tr>
<th><em>L. monocytogenes</em> strain</th>
<th>[Glucose] (mM)</th>
<th>Concentration of product (mM)</th>
<th>Carbon recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lactate</td>
<td>Formate</td>
</tr>
<tr>
<td>BHI + 10 mM glucose control*</td>
<td>10-6</td>
<td>5-0</td>
<td>9-3†</td>
</tr>
<tr>
<td>B73</td>
<td>0-03 (0-007)</td>
<td>20-9 (0-13)</td>
<td>6-4 (0-57)</td>
</tr>
<tr>
<td>B73-MR1</td>
<td>0-04 (0-04)</td>
<td>13-6 (0-59)</td>
<td>11-5 (1-72)</td>
</tr>
<tr>
<td>EGDe</td>
<td>0-04 (0-007)</td>
<td>22-0 (1-30)</td>
<td>3-4 (1-97)</td>
</tr>
<tr>
<td>EGDe-mptA</td>
<td>2-8 (0-33)</td>
<td>10-6 (0-38)</td>
<td>9-3 (1-46)</td>
</tr>
</tbody>
</table>

NB: Media concentrations of lactate, glucose and ethanol (only for EGDe-mptA) have not been subtracted from product concentrations values shown here, but are subtracted for the calculation of the carbon recovery.

*Represents the medium without bacterial inoculum.
†Indicates ethanol from the erythromycin stock used to supplement the growth of *L. monocytogenes* EGDE-mptA.
growth rate in the resistant strains when grown in the presence of glucose could be related to a lower glucose consumption rate. Clearly, EIIAB\textsubscript{Man} is not the only enzyme capable of transporting glucose since the resistant strains also consume glucose. However, this does not exclude that EIIAB\textsubscript{Man} has a control on growth rate and its elimination could affect the glucose consumption rate and specific growth rate. We took samples for glucose analysis at regular time intervals during the exponential growth phase and from a non-linear fit to the decreasing glucose concentrations with time we calculated the glucose consumption rate. The specific glucose consumption rate was subsequently calculated by normalizing the glucose consumption rate for biomass. The following specific glucose consumption rates in mmol glucose (g dry weight)\textsuperscript{−1} h\textsuperscript{−1} were determined at mid-exponential growth phase for the various \textit{L. monocytogenes} strains: B73, −15:51; B73-MR1, −6:7; EGDe, −10:73; EGDe-\textit{mptA}, −3:3. Thus, a significant reduction in the glucose consumption rate was observed for the class IIa-resistant strains in comparison to their corresponding wild-type strains.

**DISCUSSION**

The observation that the specific growth rate in class IIa bacteriocin-resistant strains, B73-MR1 and EGDe-\textit{mptA}, on media containing glucose is lower than that of the corresponding wild-type strains has also been described for another class IIa bacteriocin-resistant \textit{L. monocytogenes} strain, 412P, also showing loss of MptA expression (Gravesen \textit{et al.}, 2002a, b). The decreased growth rate in 412P, and in other class IIa bacteriocin-resistant B73 strains (Dykes & Hastings, 1998), has been interpreted as a fitness cost associated with class IIa bacteriocin resistance. This fitness cost was thought to be due to energy-expensive metabolic pathways in resistant strains (Dykes & Hastings, 1998). By taking a closer look at the physiology of these resistant strains, we can suggest a more straightforward explanation for the reduction in specific growth rate, namely the reduced consumption rate of glucose. It appears that EII\textsubscript{Man} may be the major transporter of glucose for \textit{L. monocytogenes} considering the greater than 50% decrease in glucose consumption rate observed for the resistant strains lacking MptA and evidence for the existence of only the glucose-specific enzyme IIa component and no other functional components of the glucose-specific PTS in \textit{L. monocytogenes} EGDe (Glaser \textit{et al.}, 2001). Furthermore, we suggest that the lower activity of glucose-transporting enzymes causing this extensive decrease in the glucose consumption rate is responsible for the decrease in specific growth rate.

In contrast to the results obtained in media containing glucose, we observed an increased specific growth rate for the resistant strains compared to the wild-type strains in the absence of glucose. We do not have a straightforward explanation for this result. It might be that, due to the missing glucose transporter, an up-regulation of metabolic routes for other substrates has occurred which gives these cells an advantage in the absence of glucose. An example of such an up-regulation exists for two enzymes associated with β-glucoside-specific PTSs in class IIa-resistant \textit{L. monocytogenes} strains (Gravesen \textit{et al.}, 2002b). Such an up-regulation can explain that the specific growth rate of EGDe-\textit{mptA} is largely unaffected by the availability of glucose. However, the marked decrease in specific growth rate of B73-MR1 in the presence of glucose as compared to growth on BH1 without added glucose would indicate that the regulation is repressed in the presence of glucose or that glucose has an otherwise inhibitory effect on growth rate in this resistant strain.

During our physiological characterization we noted that, in addition to the apparent disadvantage of a lower growth rate in the presence of glucose, the resistant strains had a higher biomass yield on glucose (Table 1). A product analysis revealed that the resistant strains have more of a mixed-acid type of fermentation as compared to the homolactic fermentation in the wild-type strains. In a homolactic fermentation, 2 mol ATP is formed per mole of glucose fermented and in a pure mixed-acid fermentation (i.e. no lactate formed and acetate and ethanol formed in a 1 : 1 ratio), 3 mol ATP is formed per mole of glucose fermented. The increased biomass observed in media to which glucose is added and the complete recovery of glucose in fermentation products indicates that biomass formation in our media and culture conditions is limited by the availability of the free-energy source. Thus, a shift in metabolism from a homolactic to a mixed-acid type of fermentation would result in an increase in the final biomass concentration. Quantitatively, one can check this hypothesis by calculating the biomass yield per ATP. Taking the difference in biomass formed in the presence and absence of glucose and calculating the moles of ATP formed on the basis of the product concentrations, we calculated the following biomass yields per mole ATP (Y\textsubscript{ATP}) for the four strains: B73, 9:5 g dry weight (mol ATP)\textsuperscript{−1}; B73-MR1, 8:9 g dry weight (mol ATP)\textsuperscript{−1}; EGDe, 13:0 g dry weight (mol ATP)\textsuperscript{−1}; and EGDe-\textit{mptA}, 14:8 g dry weight (mol ATP)\textsuperscript{−1}. The Y\textsubscript{ATP} values for the clinical isolate appear to be higher than those of the food isolate, but importantly the values for the resistant strains are similar to the Y\textsubscript{ATP} values of the corresponding wild-type strains. These results indicate that, apart from the changes in fermentation type, there is no apparent change in free-energy metabolism between the wild-type and the resistant strains.

A detailed mechanistic model of regulation of metabolism in \textit{Listeria} is not available at present, but a comparison to the shift from homolactic to mixed-acid fermentation in lactic acid bacteria indicates similar correlations. Lower growth rates, glucose consumption rates and glucose limitation are directly implicated in the shift from homolactic to mixed-acid fermentation (Andersen \textit{et al.}, 2001; Cocaign-Bousquet \textit{et al.}, 1996; Garrigues \textit{et al.}, 1997; Yamada & Carlsson, 1975) in \textit{Lactococcus lactis} and, although the
precise details of regulation might be different, a similar response has been observed in our studies.

*Listeria monocytogenes* has been shown to spontaneously develop resistance to class IIa bacteriocins at high frequencies from 10^{-6} to 10^{-8} in food and laboratory media (Rekhif et al., 1994; Ennahar et al., 2000b). Our results indicate that physiological responses, related to the absence of MptA in class IIa bacteriocin-resistant strains, could further compromise the potential use of class IIa bacteriocins as biopreservatives. Although resistant strains, in the presence of glucose, showed a lower specific growth rate than the wild-type strains, we have shown that the biomass yield on glucose (and potentially other energy sources) was significantly increased.

Our second finding is that together with the inactivation of the MptA a shift in metabolism occurs that could significantly alter the final concentrations of the fermentation products. Our results therefore also suggest a strong possibility that the end product of metabolism in lactic acid bacteria starter cultures could change as a result of acquiring this type of resistance to class IIa bacteriocins. It has been shown that expression of the EI56 Man in a normally insensitive *Lactococcus lactis* MG1363 strain results in the induction of sensitivity of this strain to class IIa bacteriocins (M. Ramnath, personal communication). The shift in metabolism and subsequent change in the end product would profoundly influence both the organoleptic qualities and spoilage potential of the food product.

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