Osmoprotection by carnitine in a *Listeria monocytogenes* mutant lacking the OpuC transporter: evidence for a low affinity carnitine uptake system

Katy R. Fraser a, Conor P. O’Byrne a, *a*

*Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, Scotland, UK*

Received 18 March 2002; accepted 11 April 2002

First published online 2 May 2002

Abstract

A deletion mutant of *Listeria monocytogenes* lacking OpuC, an ABC transporter responsible for the uptake of the compatible solute carnitine, was constructed and carnitine transport assays confirmed that carnitine transport was defective in this mutant. However, the mutant retained the ability to derive osmoprotection from carnitine, suggesting the presence of a second uptake system for this compatible solute. Measurement of intracellular carnitine pools during balanced growth confirmed that the opuC mutant accumulated high levels of carnitine. These pools were only achieved in the mutant when high levels (1 mM) of carnitine were present extracellularly. When a lower level (100 μM) was supplied in the medium the mutant failed to accumulate a substantial intracellular pool and failed to derive osmoprotection from carnitine. These data suggest the presence of a second low affinity carnitine uptake system in this osmotolerant pathogen. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: OpuC; Carnitine; Osmoprotection; Compatible solute; *Listeria monocytogenes*

1. Introduction

The food-borne pathogen *Listeria monocytogenes* is able to tolerate harsh environmental stresses, such as reduced water activity, and refrigeration temperatures [1,2]. Like many other organisms *L. monocytogenes* responds to conditions of hyperosmotic stress by accumulating osmotically active compounds, termed compatible solutes. The accumulation of compatible solutes acts to counteract the outward flow of water, thereby maintaining cell turgor within reasonable limits [3–5]. *L. monocytogenes* has been shown to utilise two related quaternary amine compounds as compatible solutes, l-betaine [6] and l-carnitine [7].

Verheul et al. [8] showed that carnitine accumulation in *L. monocytogenes* is mediated by a transporter with a high affinity for carnitine: a $K_m$ of 10 μM and a $V_{max}$ of 48 nmol min$^{-1}$ (mg cell protein)$^{-1}$. Transport of carnitine is independent of the osmolarity of the assay medium and is subject to negative regulation by pre-accumulated carnitine, betaine and unidentified components of peptone [8,9]. Betaine is known to be transported by at least two separate transport systems, the sodium solute symporter, BetL [10], and the ABC transporter, Gbu [11]. Betaine uptake is unaffected in the presence of 100 times excess carnitine, demonstrating that these systems are specific for betaine and do not play a role in the accumulation of carnitine [12].

Recently we reported the identification of a high affinity carnitine transport system in *L. monocytogenes*, based on homology to the OpuC transporter of *Bacillus subtilis* [13]. This transporter, designated OpuC, belongs to the binding protein-dependent ABC transporter subfamily. OpuC is encoded by a four-gene operon, *opuABCD*. The ATPase subunit is encoded by *opuCA*, the permease subunits by *opuCB* and *opuCD* and the substrate binding protein by *opuCC* [13].

A mutant of *L. monocytogenes* EGD in which the *opuC* operon has been insertionally inactivated using the suicide vector pAULA [14] is defective in its ability to transport carnitine, but not the related compounds betaine and choline [13]. Competition experiments have shown that OpuC is specific for carnitine, although competition was seen with the related compounds acetylcarnitine and γ-butyro-
betaine [8]. The opuCA::pAULA mutant shows reduced growth on complex medium with added NaCl (4% w/v) compared to the wild-type parent strain, indicating that this transporter plays an important role in osmoregulation. Unusually, the opuC::pAULA mutant strain exhibits poor growth in a defined medium, a defect that is rescued by the supplementation of the medium with peptone [13]. Here we describe the construction of an opuCA deletion strain and present evidence for a previously unidentified low affinity carnitine transporter in *L. monocytogenes*.

2. Materials and methods

2.1. Strains and growth experiments

The wild-type *L. monocytogenes* serotype 1/2a strain EGD and a ΔopuCA derivative of this strain were used throughout. Growth experiments were carried out in defined medium (DM), as previously described [15]. The medium osmolarity was raised by the addition of NaCl. Experiments were carried out in duplicate on the day, and repeated independently at least twice. Representative growth data are shown, whilst specific growth rates are described as means ± S.D.

2.2. Construction of opuCA deletion mutant

An in-frame deletion mutant was constructed by PCR (splicing by overlap extension) SOEing, as described previously [16]. The oligonucleotide primers used to generate the deletion were as follows: P160 (5′-cgccttatcttatttacagct-taagtgtggag-3′), P124 (5′-ttaacctggtggctatctgattg-3′), P161 (5′-ggctgttctgctagtcgccggtggta-3′) and P111 (5′-ctttccttgctccatcaagttcaaggg-3′). Primer 160 included an 18-bp tail, complementary to 18 bp of primer 161 (underlined sequence). The suicide vector pKSV7 was used to introduce the *opuCA* deletion into EGD, as described previously [17]. The presence of the chromosomal *opuCA* deletion was confirmed by PCR using primers P130 (5′-gattttgacycgctgacaag-3′) and P123 (5′-cgtaggtggagaagcag-3′). The 2.1-kb PCR product resulting from this reaction was sequenced on both DNA strands and the presence of an in-frame 1-kb deletion in the *opuCA* gene (removing amino acid residues 25–321 from the OpuCA protein) was confirmed. The absence of vector sequences in the deletion mutant was confirmed by PCR using primers Pfor (5′-tgtaaaagcagcgccagt-3′) and Prev (5′-cagggagacagctatgac-3′).

2.3. Carnitine transport assays

The uptake of carnitine was measured as previously described [13]. When measuring transport in the presence of differing concentrations of carnitine, secondary stock solutions were created with unlabelled carnitine to give a final carnitine concentration of 100 μM or 1 mM when added to the cell suspension. [1H]Carnitine-HCl (Amersham Pharmacia Biotech) was added to this secondary stock at a fixed specific activity (29.9 μCi μmol⁻¹). The rate of carnitine accumulation was determined by calculating the slope of the line of the linear part of the uptake curve. The total cell protein values used to calculate solute pools were 170 μg ml⁻¹ OD₆₀₀ unit⁻¹ at low osmolarity and 130 μg ml⁻¹ OD₆₀₀ unit⁻¹ at high osmolarity, as previously described [15].

2.4. Measurement of steady-state solute pools

Steady-state cytoplasmic carnitine pools were determined as previously described [13]. For measurement of pools in the presence of 1 mM or 100 μM carnitine, carnitine at these concentrations was added to the growth media at a fixed specific activity of 300 nCi μmol⁻¹ of [3H]carnitine-HCl.

3. Results

3.1. Construction of opuCA deletion mutant

Previously we reported the generation of an opuC::pAULA disruption mutant, which failed to grow in DM, a phenotype that was rescued by the addition of peptone to the growth media [13]. In subsequent experiments we found that *L. monocytogenes* EGD (pAULA) displayed a similar growth defect in DM (data not shown). To resolve this problem a deletion mutant lacking opuCA, the first gene of the opuC operon, was constructed as described in Section 2.

In order to confirm that the ΔopuCA mutant was deficient in carnitine transport, the ability of the ΔopuCA cells to accumulate carnitine was investigated using tritiated ([3H] carnitine (20 μM). In wild-type cells carnitine is accumulated at a rate of ~120 nmol min⁻¹ (mg cell protein)⁻¹, regardless of the presence or absence of added NaCl (Fig. 1), confirming previous findings that carnitine uptake is not salt activated [8,9]. In the ΔopuCA mutant carnitine transport is almost entirely abolished, with only a small amount of residual activity remaining (~1 nmol min⁻¹ (mg cell protein)⁻¹). These data confirmed that the opuCA deletion disrupted the activity of the major carnitine transporter in *L. monocytogenes*.

The ΔopuCA mutant was tested for its ability to grow in DM. The deletion mutant grew with a similar specific growth rate to the wild-type (0.45 h⁻¹ and 0.47 h⁻¹, respectively), suggesting that the growth defect in DM observed for the opuC::pAULA mutant was not due to the lack of the OpuC transport system.
3.2. The \( \Delta \text{opuCA} \) mutant can use carnitine as an osmoprotectant

We investigated the ability of the \( \Delta \text{opuCA} \) mutant strain to utilise carnitine as an osmoprotectant in DM, an experiment we were unable to perform with the \( \text{opuCA}:\text{pAU-LA} \) mutant because of its impaired growth in DM [13]. This was done by measuring growth at increased osmolarities in the presence or absence of carnitine. There was no difference in the growth rate between the wild-type and \( \Delta \text{opuCA} \) strain in DM. There was a reduction of approximately 50% in growth rate of both wild-type and mutant strains in the presence of 0.5 M NaCl (Fig. 2). The addition of 1 mM carnitine to DMS resulted in increased growth in both strains. The specific growth rates of wild-type and \( \Delta \text{opuCA} \) mutant were identical, 0.26 (\( \pm 0.01 \)) h\(^{-1}\) and 0.26 (\(< 0.01\)) h\(^{-1}\), respectively, representing an increase of approximately 25% in growth rate (Fig. 2). The wild-type and mutant behaved similarly when 0.8 M NaCl was used to raise the medium osmolarity (data not shown). These data show that cells lacking \( \text{opuCA} \) retain the ability to utilise carnitine as an osmoprotectant. This result was surprising since we expected the disruption of a major carnitine transport system to lead to a loss of osmoprotection by carnitine.

3.3. Carnitine accumulation in the \( \Delta \text{opuCA} \) mutant

To determine how the \( \Delta \text{opuCA} \) deletion affected the accumulation of carnitine in growing cells, the cytoplasmic carnitine pools during balanced growth in DM with 1 mM carnitine, with or without 0.5 M NaCl, were measured. In the absence of added salt the \( \Delta \text{opuCA} \) mutant possesses a carnitine pool approximately half the size of the wild-type. When cells are grown in the presence of 0.5 M NaCl, both the wild-type and \( \Delta \text{opuCA} \) mutant cells show a large increase in the cytoplasmic carnitine pool (Fig. 3A). Although the final pool in the \( \Delta \text{opuCA} \) mutant is reduced compared to the wild-type (1031 nmol (mg cell protein\(^{-1}\)) and 1549 nmol (mg cell protein\(^{-1}\)), respectively), clearly this substantial pool is sufficient to allow the osmoprotection observed under the growth conditions tested (Fig. 2). These data pointed to the presence of an additional carnitine uptake system, that could compensate for the lack of functional OpuC in the presence of high extracellular carnitine concentrations (1 mM).

Next we investigated whether this alternative carnitine uptake system could generate a cytoplasmic pool of carnitine when the extracellular concentration of this compatible solute was lowered from 1 mM to 100 \( \mu \text{M} \). The wild-type cells accumulated pools similar in size to those accumulated in the presence of 1 mM carnitine, suggesting that carnitine was not limiting at this concentration. However, the intracellular pool of carnitine accumulated by the \( \Delta \text{opuCA} \) mutant was reduced significantly compared to the pool observed in the mutant growing in the presence of 1 mM extracellular carnitine (Fig. 3A,B). The addition of 0.5 M NaCl led to a 6-fold increase in pool size, but the steady-state pool size was still approximately 90% lower than the wild-type in the same medium (168 nmol (mg cell protein\(^{-1}\)) and 1940 nmol (mg cell protein\(^{-1}\)), respectively) (Fig. 3B). These data show that the size of the carnitine pool is dependent on the carnitine concentration in the growth medium and point to the presence of a previously uncharacterised low affinity carnitine transport system in \( \text{L. monocytogenes} \).

The existence of a low affinity uptake system is also
suggested by measurements of the rate of carnitine transport in the presence of either 1 mM or 100 μM. The rate of carnitine transport seen by the wild-type strain is not affected by the reduction in substrate concentration from 1 mM to 100 μM carnitine (Fig. 4A,B), consistent with the known high affinity of OpuC for carnitine (apparent $K_m$ is approximately 10 μM [8]). However, the $\Delta$opuCA strain only shows detectable uptake of carnitine in the presence of 1 mM carnitine (Fig. 4A,B), suggesting that the remaining uptake system has a lower affinity for this compatible solute than OpuC.

3.4. Carnitine transport in the absence of OpuC is insufficient for osmoprotection in the presence of low carnitine concentrations

The transport data above suggested a possible explanation for the ability of 1 mM carnitine to act as an osmo-proteactant when the $\Delta$opuCA mutant was in DM with 0.5 M added NaCl (Fig. 2); that is, the low affinity uptake system(s) present in the mutant could accumulate sufficient carnitine to allow osmoprotection and therefore an enhanced growth rate. To test this we performed a growth
experiment where the wild-type and ΔopuCA mutant were grown in high osmolarity medium (DM, 0.5 M NaCl) with or without 100 μM carnitine. Under these conditions the wild-type showed an increased growth rate (approximately 20%) compared to the control, which had no added carnitine. However, the ΔopuCA mutant was unable to use this low concentration of carnitine as an osmoprotectant and no significant increase in the growth rate was detected (Fig. 5).

4. Discussion

Here we have described the construction and characterisation of an in-frame deletion mutant of *L. monocytogenes* which lacks the ATPase subunit of the OpuC transporter, a high affinity carnitine uptake system that belongs to the ABC transporter superfamily. The ΔopuCA mutant grows normally in our DM and there is no evidence of impaired growth even when the osmolarity of the growth medium is raised with 0.5 M NaCl (Fig. 2). This is in contrast to an insertion mutant (Δ*opuCA*::pAULA) that we have described previously; this mutant failed to grow in DM, a phenotype that we attributed to the loss of the OpuC transporter, as well as to compatible solute binding subunit, respectively. Further experiments are underway to determine if these genes encode the low affinity carnitine transport system that we have described here.

Acknowledgements

The authors wish to thank N. Freitag for supplying the pKSV7 suicide vector. We would also like to thank P. Glaser for sharing information prior to publication and Ian Booth for useful discussions. K.R.F. is supported by a Unilever BBSRC CASE studentship.
References


