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ORIGINAL PAPER

Gluconeotrehalose is the principal organic solute in the psychrotolerant bacterium *Carnobacterium* strain 17-4

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Abstract A high proportion of microorganisms that colonise cold environments originate from marine sites; hence, they must combine adaptation to low temperature with osmoregulation. However, little or nothing is known about the nature of compatible solutes used by cold-adapted organisms to balance the osmotic pressure of the external medium. We studied the intracellular accumulation of small organic solutes in the Arctic isolate *Carnobacterium* strain 17-4 as a function of the growth temperature and the NaCl concentration in the medium. Data on 16S rDNA sequence and DNA–DNA hybridisation tests corroborate the assignment of this isolate as a new species of the bacterial genus *Carnobacterium.* The growth profiles displayed maximal specific growth rate at 30°C in medium without NaCl, and maximal values of final biomass at growth temperatures between 10 and 20°C. Therefore, *Carnobacterium* strain 17-4 exhibits halotolerant and psychrotolerant behaviours. The solute pool contained glycine-betaine, the main solute used for osmoregulation, and an unknown compound whose structure was identified as α-glucopyranosyl-(1-3)-β-glucopyranosyl-(1-1)-α-glucopyranose (abbreviated as gluconeotrehalose), using nuclear magnetic resonance and mass spectrometry. This unusual solute consistently accumulated to high levels (0.35 ± 0.05 mg/mg cell protein) regardless of the growth temperature or salinity. The efficiency of gluconeotrehalose in the stabilisation of four model enzymes against heat damage was also assessed, and the effects were highly protein dependent. The lack of variation in the gluconeotrehalose content observed under heat stress, osmotic stress, and starvation provides no clue for the physiological role of this rare solute.

Keywords Cold-adapted bacterium · Osmoregulation · α,β-Trehalose · Enzyme stabilisation · Trisaccharide

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Introduction

One of the most common strategies used by micro-organisms to cope with environmental variations in water activity is the accumulation of compatible solutes inside the cell (da Costa et al. 1998; Wood et al. 2001). According to the initial description by Brown (1976), these are small organic solutes with no net charge that can accumulate to high levels in the cytoplasm with minimal metabolic perturbation. Thus, by controlling the intracellular water activity, this strategy allows cells to maintain turgor, a prerequisite for cell viability. But compatible solutes appear to play a variety of functions besides maintaining osmotic balance. In fact, many organisms subjected to heat

stress also accumulate small organic solutes which have the ability to protect cellular structures and, in particular, to enhance protein thermostability (de Virgilio et al. 1994; Lamosa et al. 1998; Santos and da Costa 2001; Kandror et al. 2002; Faria et al. 2008; Santos et al. 2011). Among model mesophiles, *Saccharomyces cerevisiae* is a paradigm insofar as huge amounts of trehalose accumulate in response to heat stress (Hottiger et al. 1987).

With this in mind, it is reasonable to question whether the environmental temperature for optimal growth of an organism influences the nature of the organic solutes preferred for osmotic balance. While mesophiles tend to accumulate uncharged or zwitterionic compounds, such as polyols, non-reducing sugars, and amino acids (da Costa et al. 1998), hyperthermophilic organisms, thriving at temperatures above 80°C, have a tendency to accumulate charged solutes, such as polyolphosphodiesters and anionic sugar derivatives (Santos et al. 2011). Furthermore, the solutes typically found in organisms adapted to hot environments appear to be more effective than their mesophilic counterparts in the protection of proteins from thermal denaturation (Ramos et al. 1997; Lamosa et al. 2003; Faria et al. 2004).

By analogy, at the other end of the growth temperature scale, psychrophiles, organisms able to grow at temperatures around the freezing point of water, may have evolved to accumulate specific solutes best suited for the requirements of life at low temperature. This hypothesis lacks experimental support given the scarcity of data on solute accumulation by cold-adapted organisms (Cavicchioli et al. 2000; Cavicchioli 2006). To our knowledge, there are only two reports on solute accumulation in psychrophiles, both on the archaeon *Methanococcoides burtonii* (Thomas et al. 2001; Costa et al. 2006).

To expand our knowledge and investigate the possible special properties of compatible solutes from cold environments, we decided to study the organic solute content of the arctic isolate *Carnobacterium* strain 17-4 as a function of the growth temperature and the NaCl concentration in the growth medium. This bacterium was isolated from seawater in the permanently cold fjords of Spitzbergen (Norway), where the salt concentration varies from zero (fresh water) to that of sea water, thus making it a good subject for studying osmoregulation strategies in cold-adapted organisms.

Materials and methods

Materials

Mitochondrial malate dehydrogenase (MDH) from pig heart was purchased from Roche; porcine liver esterase was from Sigma. For the calorimetry assays, hen egg lysozyme was purchased from Fluka, and *Staphylococcus aureus* nuclease A (SNase) was produced and purified from *E. coli* as described by Faria et al. (2004); for the thermoinactivation assays, SNase was obtained from Sigma and lysozyme from AppliChem. *p*-Nitrophenyl butyrate (*p*-PNB) and *Micrococcus lysodeikticus* cells were purchased from Sigma. All other materials were reagent grade.

Enrichment, isolation, and cultivation

Seawater samples were collected in the permanently cold fjords of Spitsbergen, Norway. Part of a membrane filter (cellulose acetate, pore size 0.2 μm), through which seawater was filtered, or 0.5 ml of untreated liquid sample was used for inoculation of 10 ml of complex marine medium in 20-ml tubes. Complex marine medium (EMP-2 medium; Groudieva et al. 2004) consisted of basal medium supplemented with a solution of different carbon sources, which was sterilised by filtration and contained (final concentration, per litre): 0.5 g Na-acetate, 0.5 g Na_2-succinate, 0.5 g DL-malate, 0.5 g Na-pyruvate, 0.5 g D-mannitol, and 2.0 g glucose. The basal medium contained (per litre): 28.13 g NaCl, 0.77 g KCl, 0.02 g $CaCl_2{\cdot}2H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, 1.0 g NH_4Cl, 0.02 g iron-ammonium-citrate, 0.5 g yeast extract, 10 ml trace element solution (DSM 141), 10 ml vitamin solution (DSM 141), 2.3 g KH_2PO_4, 2.9 g $Na_2HPO_4{\cdot}2H_2O$. The pH was adjusted with NaOH to 7.2. After 2 months, stable enrichment cultures have developed at 4°C and were serially diluted onto EMP-2 medium agar-plates. Plates were incubated at 4°C for 2 months before colonies were selected on the basis of morphological differences. For the isolation of pure cultures, serial dilution and plating techniques were applied. Isolates were routinely cultivated on EMP-2 agar-plates at 4°C.

16S rDNA amplification and sequencing

Biomass from a single colony or 1 μl of cell pellet obtained by centrifugation of 1 ml of exponentially growing cultures was used directly as template for the amplification of 16S rDNA. The 16S rDNA gene was amplified with 9-27f and 1492-1515r 16S rDNA primers (Buchholz-Cleven et al. 1997). Each 100-μl PCR reaction mixture contained 50 pmol of each primer, 1–50 ng template DNA, 200 μM dNTP each, 30 μg bovine serum albumin, 20 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 U of *Taq* polymerase (Gibco). Negative control without DNA template was included in every reaction set. All reaction mixtures were incubated in a thermal cycler (PerkinElmer Gene Amp System 2400) for 5 min at 94°C and then subjected to 30 amplification cycles of 1.5 min at 94°C,

1.5 min at 44°C, and 1.5 min at 72°C. The amplicons were separated on 1% (w/v) agarose gel stained with ethidium bromide. 16S rDNA genes were partially sequenced. The *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) was used to directly sequence the purified PCR product (QIAquick PCR purification kit, Qiagen). Sequencing reactions were analysed on the Applied Biosystems 373S DNA sequencer. Forward primers 341F and 518F (Buchholz-Cleven et al. 1997) were used for partial sequence analysis. For the complete sequence analysis, both strands of the amplification product were sequenced also using the primers 7F, 787F, 787R, 1175R, 1099F, and 1492R (Buchholz-Cleven et al. 1997). Primer nomenclature refers to the 5′ ends of respective target on the 16S rDNA according to *Escherichia coli* numbering of 16S rRNA nucleotides. Obtained sequences were used for phylogenetic analysis. To determine the closest relatives of the new isolate, preliminary searches in the EMBL database were performed with the programme FASTA3. The DNADIST programme, with the maximum-likelihood option, was employed to analyse sequence similarities.

DNA–DNA hybridisation and determination of DNA G + C content

DNA–DNA hybridisation reactions and DNA G + C content determination were performed at the DSMZ (Braunschweig, Germany). Genomic DNA samples from *Carnobacterium* strain 17-4, *Carnobacterium viridans* DSM 14451, and *Carnobacterium alterfunditum* DSM 5972 were used for DNA–DNA hybridisation. DNA G + C content was analysed by HPLC according to the methods of Mesbah et al. (1989) and Tamaoka and Komagata (1984).

Growth conditions for solute quantification

Carnobacterium strain 17-4 was grown in 2-l Erlenmeyer flasks containing 0.5 l of EMP-2 medium (Groudieva et al. 2004) with 2.0 g of glucose and 0.5 g of each of the following substrates: sodium acetate, sodium succinate, sodium pyruvate, mannitol, and malic acid. Growth was achieved under 180 rpm shaking, at different temperatures (10, 15, and 20°C) and with different salt concentrations [0, 2, 2.8, 4, 6, 8, and 10% NaCl (w/v)]. Additional temperatures (25, 30, and 37°C) were examined, but only in medium with no added NaCl. Growth was evaluated by measuring optical density at 600 nm.

Intracellular solute extraction and quantification

Cells were harvested by centrifugation (4000×*g*, 7 min) during the mid-exponential growth phase. Total protein content was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA) after cell lyses by sonication and treatment with 0.1 M NaOH. Intracellular solutes were extracted from the cell mass twice with boiling ethanol followed by chloroform treatment according to the method described by Martins and Santos (1995). Extracts were dissolved in a known amount of deuterated water and analysed by NMR.

Production, purification, and acid hydrolysis of the oligosaccharide

For characterisation of the unknown solute, *Carnobacterium* strain 17-4 was grown under osmotic stress in EMP-2 medium (Groudieva et al. 2004) containing 8% (w/v) of NaCl at 15°C in a 300-l fermentor. The cells were harvested by centrifugation (4000×*g*, 7 min) at an optical density of 0.6. A quantity of 215 g of biomass was obtained, from which approximately 12 g of the compound was extracted. The cell extracts were applied onto a QAE-Sephadex ion exchange column (Pharmacia, Uppsala, Sweden) equilibrated with 5 mM sodium carbonate buffer (pH 9.8). The non-ionic fraction of this column (flow-through) was freeze-dried and loaded onto an activated Dowex 50W-X8 column to remove buffer salt. The compound was eluted with distilled water, and the pH of the eluate was adjusted to 7 with KOH. After freeze-drying, the compound was judged pure by ^{1}H-NMR analysis. An amount of 8.3 g of the pure trisaccharide was obtained after purification. To identify the unit constituents of the polysaccharide, a sample was hydrolysed with 0.5 M H_2SO_4 at 100°C for 2 h under an argon atmosphere in a sealed ampoule and subsequently neutralised to pH 7.2 with NaOH.

NMR spectroscopy

All spectra were acquired on a Bruker DRX500 spectrometer (Bruker, Rheinstetten, Germany). ^{13}C-NMR spectra were recorded at 125.77 MHz using a 5-mm carbon selective probe head. Typically, spectra were acquired with a repetition delay of 1.5 s and a pulse width of 7 μs, corresponding to a 70° nutation angle. When required, proton decoupling was applied during the acquisition time. Chemical shifts are referenced to the methyl resonance of 3-(trimethylsilyl)propanesulphonic acid (sodium salt) at 0.0 ppm.

^{1}H-NMR spectra were acquired at 500.13 MHz in a 5-mm inverse detection probe head. Water presaturation was applied. Chemical shifts are relative to 3-(trimethylsilyl)propanesulphonic acid (sodium salt). For quantification purposes, a known amount of formate was added, and a repetition delay of 60 s was used to ensure full relaxation of the signals.

Two-dimensional spectra were performed using standard Bruker pulse programmes. Phase-sensitive nuclear Overhauser effect spectroscopy (NOESY), proton-homonuclear correlation spectroscopy (COSY), and total-correlation spectroscopy (TOCSY) were acquired in 4096 (t_2) × 512 (t_1) data points; in ^{1}H–^{13}C heteronuclear multiple quantum coherence (HMQC) spectra, a delay of 3.5 ms was used for evolution of $^{1}J_{CH}$. The heteronuclear multiple bond connectivity (HMBC) spectra were recorded by collecting 4096 (t_2) × 256 (t_1) data points; a delay of 73.5 ms was used for evolution of long range couplings.

Derivatisation of the trisaccharide

Signal overlapping in important regions of the NMR spectra of the compound caused some assignment difficulties. Therefore, we decided to acetylate all free hydroxyl groups, a strategy that caused the shift of some of the resonances and allowed for the firm elucidation of the position of the glycosidic bonds and of the full structure.

A sample of the lyophilised compound (133 mg) was added to 374 µl of dried acetic anhydride and a catalytic amount of dimethylaminopyridine in 3 ml of pyridine with stirring under argon. After 24 h, the reaction was judged complete by TLC, and sodium bicarbonate in water was added to exhaust the excess anhydride. The product was extracted three times with ethyl acetate, dried with anhydrous magnesium sulphate and filtered. The solvent was removed and the product dissolved in a small amount of dichloromethane where it began to crystallise, yielding a white solid (233 mg, 92%).The acetylated compound was dissolved in deuterated DMSO for NMR analysis and a set of one-dimensional ^{1}H and ^{13}C and two-dimensional COSY, NOESY, TOCSY, HMQC and HMBC spectra were acquired as described above.

Differential scanning calorimetry

The assays were performed on a MicroCal VP-DSC Microcalorimeter (MicroCal, Northampton, MA, USA). The effect of the trisaccharide on the melting temperature of nuclease A from *Staphylococcus aureus* (SNase), malate dehydrogenase (MDH) from pig heart, esterase from porcine liver, and lysozyme from hen egg white was studied by differential scanning calorimetry and compared with the common stabiliser, trehalose. For SNase and lysozyme, the protein final concentration was approximately 20 µM; esterase was used at 30 µM and MDH at 5 µM. All solutes were used at a final concentration of 0.5 M. After degassing under vacuum for 8 min, samples were heated from 25 to 90°C at a heating rate of 1°C/min. Thermograms were analysed using standard MicroCal software (MicroCal, Northampton, MA, USA).

Thermostability assays

The thermostability of the four selected enzymes was assessed in the presence or absence of 0.5 M gluconeotrehalose or trehalose. Esterase activity was measured by a spectrophotometric assay with *p*-nitrophenyl butyrate (*p*-PNB) as substrate. Cleavage of *p*-PNB (Sigma, Germany) was determined at 37°C in 50 mM Tris–HCl buffer pH 7.0, according to Winkler and Stuckmann (1979). Sample blanks were used to correct the non-enzymatic cleavage of *p*-PNB. The thermostability of esterase was evaluated by measuring the residual activity of the enzyme after 5, 10, or 20 min of incubation at 70°C.

Nuclease activity was determined spectrophotometrically at 600 nm by measuring the increase in turbidity induced by acidification of a DNA solution according to the method by Erickson and Deibel (1973). The thermostability of nuclease was evaluated by measuring the residual activity of the enzyme after 10, 20, or 30 min of incubation at 99°C.

Lysis of *Micrococcus lysodeikticus* cells (Sigma, Germany) was determined at 25°C for 10 min in 66 mM potassium phosphate buffer (pH 6.24) according to the method by Shugar (1952). The reaction was terminated by placing the samples on ice, and the absorbance was measured at 450 nm. The thermostability of lysozyme was evaluated by measuring the residual activity of the enzyme after 10, 20, or 30 min of incubation at 80 and 90°C.

The MDH activity was assayed spectrophotometrically at 25°C following the conversion of NADH to NAD^{+} at 340 nm as described by Jorge et al. (2007). The enzyme was incubated at 45°C, and the time courses for thermal inactivation were fitted to single exponential decays.

Mass spectrometry

Mass spectra were acquired on a LCQ advantage ion trap mass spectrometer from ThermoFinnigan (San Jose, CA, USA) equipped with an electrospray ionisation interface operated in the negative mode. Samples were injected at 300°C and -33 V in 50% methanol/0.1% formic acid.

Results

Classification of the organism

Strain 17-4 was isolated from seawater samples collected at the fjord of Spitsbergen (Groudieva et al. 2004), and the 16S rDNA gene sequence of the isolated strain was compared with the EMBL nucleotide database by basic local alignment. The sequence of strain 17-4 showed the highest similarity (98.8%) to the sequence of *Carnobacterium*

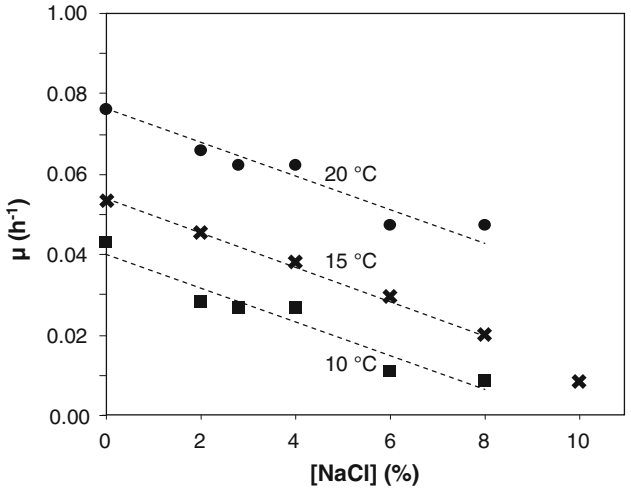

Fig. 1 Specific growth rate of *Carnobacterium* strain 17-4 cells at different temperatures (*squares* 10°C, *crosses* 15°C, *circles* 20°C) and different salt concentrations (0, 2.0, 2.8, 4.0, 6.0, 8.0, and 10% NaCl). Growth profiles were also studied at 10 and 20°C in medium containing 10% NaCl, but growth was poor, precluding a reliable determination of the specific growth rate. Data are averages of two independent experiments

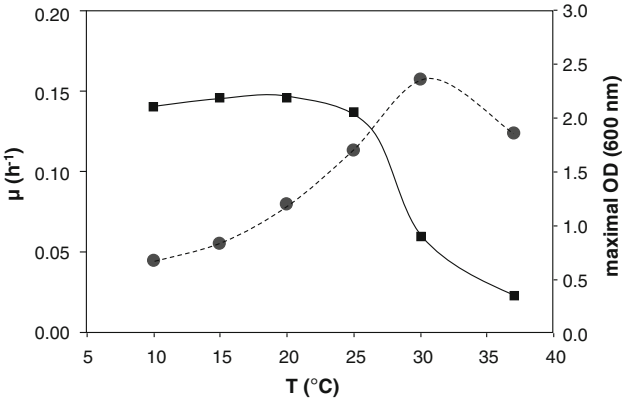

Fig. 2 Specific growth rate (*circles*, *left-hand axis*) and maximal optical density (*squares*, *right-hand axis*) of *Carnobacterium* strain 17-4 cultures as a function of the growth temperature. The growth medium contained no added NaCl. Data are averages of two independent experiments

alterfunditum. The DNA G + C content of *Carnobacterium* strain 17-4 was 28 mol%. DNA–DNA hybridisation tests revealed relatedness values of *Carnobacterium* strain 17-4 of 28.5 and 26.5% with *C. alterfunditum* DSM 5972 and 20.3 and 17.4% with *C. viridans* DSM 14451, respectively. These values were well below the recommended threshold value of 70% for the assignment of novel species (Wayne et al. 1987).

Effect of salinity and temperature on growth

The dependence of the specific growth rate with the concentration of NaCl (0–10%) in the medium was studied at 10, 15, and 20°C (Fig. 1). The growth rate decreased monotonically with the concentration of NaCl in the growth medium, indicating typical halotolerant behaviour. It is noteworthy that this non-halophilic bacterium was able to grow at relatively high salinities (up to 10% NaCl, at 15°C).

In the absence of added NaCl, the specific growth rate increased with temperature up to a maximum of 0.156 h^{-1} at 30°C (Fig. 2). Curiously, maximal turbidity values ($OD_{600} \approx 2$) were obtained at temperatures between 10 and 25°C, and the final OD_{600} dropped sharply at temperatures above 25°C. In other words, although this bacterium exhibited maximal specific growth rate at 30°C, the final cell density at this temperature was poor and much lower than that observed at cooler temperatures (Fig. 2). Absolute, maximal turbidity values ($OD_{600} = 2.5$) were found at 15°C with 2% NaCl concentration (data not shown).

If we consider the specific growth rate as the most reliable indicator of growth efficiency, then 30°C is the optimal temperature for *Carnobacterium* strain 17-4. While it is true that cells grew faster at 30°C, it is also true that the exponential phase was rather short and there is clear evidence for cell lysis at the early stationary phase and afterwards, contrary to what happens at lower temperatures. These features were aggravated at 37°C (Fig. 1S). Therefore, we think that the final cell density is a better parameter to indicate the most favourable conditions for growth of *Carnobacterium* strain 17-4.

Identification of organic solutes

^{1}H-NMR spectra of ethanol extracts of *Carnobacterium* strain 17-4 showed several sets of resonances that were assigned to alanine, lactate, glycine-betaine, and an unidentified compound, presenting three signals in the anomeric region. The firm identification of these compounds was achieved by ^{13}C-NMR through comparison with the carbon chemical shift values reported in the literature. The remaining set of resonances in the ^{13}C-NMR spectrum (105.5; 102.8; 101.9; 84.6; 78.6; 75.6; 75.5; 75.4; 74.6; 74.4; 72.5; 72.1; 71.9; 63.2; 62.8 ppm) could not be assigned to a known compound. This unknown compound was purified from the cell extract by column chromatography. Resonances at 105.5, 102.8, and 101.9 ppm were assigned to three anomeric CH groups, and the question arose whether the compound was a trisaccharide or a higher polysaccharide with a trisaccharide-repeating unit. A molecular mass of 504 was determined by mass spectrometry corresponding to the mass of three hexose moieties linked by two glycosidic bonds. Acid hydrolysis of the

compound yielded glucose as the sole product. At this stage, a set of COSY, NOESY, TOCSY, HMQC, and HMBC experiments were run to establish the structure of the trisaccharide. The 2D-TOCSY experiment presented three spin-systems corresponding to the three glucose moieties with patterns of $^{3}J_{H,H}$ coupling constants characteristic of glucopyranoses, two of them in the α and one in the β configuration. The homonuclear proton COSY experiment allowed the unequivocal assignment of all the individual proton signals belonging to the two moieties in the α configuration and of the signals belonging to protons 1, 2, 3, and 6 of the β moiety. The HMQC spectrum (Fig. 2S) permitted to assign the resonances of the carbon spectrum. This spectrum correlated three proton anomeric resonances at 5.33, 5.23, and 4.66 ppm with the carbon signals at 101.9, 102.8, and 105.5 ppm, respectively, and was annotated as Gα1, Tα1, and Tβ1, respectively. These carbon signals belonging to anomeric CH groups presented $^{1}J_{C,H}$ values of 172.6, 172.3, and 161.0 Hz, respectively, confirming the two α and one β configurations.

The NOESY spectrum showed a clear correlation between the anomeric protons at 5.23 and 4.66 ppm ($H_{T\alpha1}$ and $H_{T\beta1}$). This linkage was further confirmed in the HMBC experiment, thus establishing the presence of an α,β-trehalose moiety. The compound was thus identified as an α-glucosyl-α,β-trehalose. The NMR chemical shifts are presented in Table 1.

At this stage, the only structural question left to answer was the location of the α linkage between position 1 of the glucosyl moiety and the α,β-trehalose. The NOESY spectrum showed correlations between Gα1 and a signal at 3.66 ppm belonging to the Tβ moiety, where the resonances of $H_{T\beta3}$ and $H_{T\beta5}$ occur. As the hydroxyl group at position 5 is occupied by the bond responsible for the pyranose ring, this linkage can only be formed between Gα1 and Tβ3. In the light of these results, the compound was identified as α-glucopyranosyl-(1-3)-β-glucopyranosyl-(1-1)-α-glucopyranose (Fig. 3).

This identification was further confirmed by the analysis of the spectra of the acetylated compound. After acetylation, the ^{1}H spectrum became considerably different not only due to new resonances in the methyl region but also because acetylated groups were shifted towards lower field. The ^{13}C spectrum, however, presented small shifts, with the anomeric carbons very close to their original positions. This allowed us to use the $^{1}H/^{13}C$-HMQC spectrum to assign the positions of the anomeric protons. The combined use of the COSY and TOCSY spectra allowed identifying all the resonances belonging to glucosyl moieties. The NOESY spectrum revealed connectivities between the anomeric protons $H_{T\alpha1}$ and $H_{T\beta1}$, thus confirming the α,β-trehalose moiety and a correlation between $H_{G\alpha1}$ and $H_{T\beta3}$ firmly establishing the α(1→3) glycosidic linkage between the glucose residue and the β moiety of the α,β-trehalose. Hereafter, the compound will be designated gluconeotrehalose for the convenience of a short name.

Table 1 ^{13}C and ^{1}H chemical shifts (ppm) of α-glucopyranosyl-(1-3)-β-glucopyranosyl-(1-1)-α-glucopyranose measured at 25°C in deuterated water (pH 6.4)

	Moiety		
	α-glucosyl (Gα)[a]	β-glucosyl (Tβ)[a]	α-glucosyl (Tα)[a]
C1	101.9	105.5	102.8
H1	5.33	4.66	5.23
C2	74.4	78.6	74.4
H2	3.56	3.48	3.56
C3	75.6	84.6	75.5
H3	3.74	3.66	3.73
C4	71.9	74.6	72.1
H4	3.47	3.50	3.44
C5	74.4	72.5	75.4
H5	4.02	3.64	3.93
C6	62.8	63.2	63.2
H6	3.79	3.86; 3.75	3.83; 3.71

[a] Designation of glucose units according to Fig. 3

Effect of salinity and temperature on organic solute accumulation

Gluconeotrehalose and glycine-betaine were the two major solutes accumulating in *Carnobacterium* strain 17-4 (Figs. 4, 5; Table 1S). An increase in the salinity of the growth medium from 0 to 8% NaCl led to an increase of glycine-betaine, while the level of gluconeotrehalose remained fairly constant throughout this salinity range and at all the temperatures examined (10, 15, and 20°C). For example, at 20°C, the intracellular concentration of glycine-betaine increased progressively from 0.03 to 0.85 μmol mg protein^{-1} with the salinity of the growth

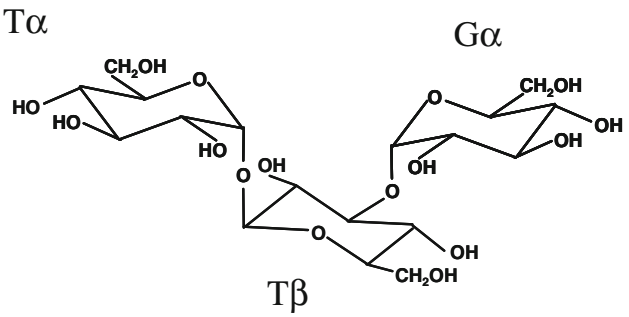

Fig. 3 Structural representation of α-glucopyranosyl-(1-3)-β-glucopyranosyl-(1-1)-α-glucopyranose (gluconeotrehalose). Each glucosyl unit is labelled according to the convention used in the assignment of the NMR resonances

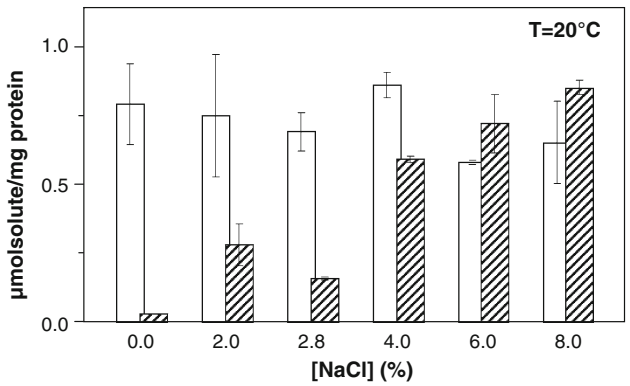

Fig. 4 Accumulation of solutes in *Carnobacterium* strain 17-4 as a function of the NaCl concentration in the growth medium. Cells were cultivated at 20°C, harvested during mid-exponential phase and treated with ethanol-chloroform for extraction of solutes, which were quantified by proton NMR. *Open bars* gluconeotrehalose, *stippled bars* glycine-betaine. Data are averages of two independent experiments

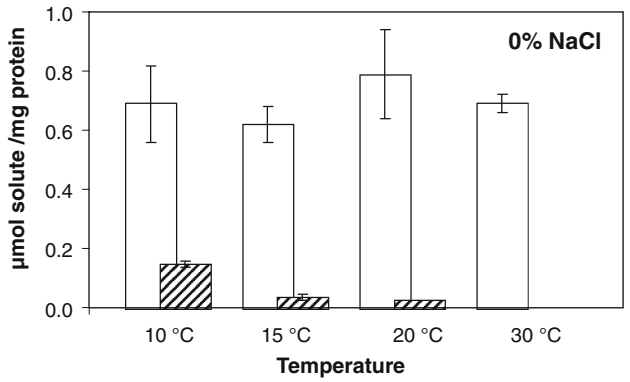

Fig. 5 Accumulation of solutes as a function of the growth temperature in *Carnobacterium* strain 17-4 cultivated in medium without added NaCl. Cells were harvested during mid-exponential phase and treated with ethanol–chloroform for extraction of solutes, which were then quantified by proton NMR. *Open bars* gluconeotrehalose, *stippled bars* glycine-betaine. Data are averages of two independent experiments

medium, while gluconeotrehalose fluctuated around the average value of 0.7 ± 0.1 µmol mg protein^{-1}, with no apparent trend (Fig. 4). The same average content of gluconeotrehalose was observed in cells grown at temperatures between 10 and 30°C in medium without added NaCl (Fig. 5).

To investigate whether gluconeotrehalose was utilised as a carbon reserve, cells were grown at 20°C in the medium described above, without added NaCl, and harvested at $OD_{600} = 1.5$ (stationary phase). Cells were washed with medium lacking carbon nutrients, suspended in this medium, and incubated for 18 h under the same conditions used for growth. At time points 0, 6, 12 and 18 h, samples were collected and analysed in respect to solute content. There was no significant change in the level of gluconeotrehalose or glycine-betaine (results not shown).

Effect of gluconeotrehalose on the melting temperature of enzymes

The thermograms of MDH, SNase, and lysozyme show single transitions at 48.3, 54.2, and 72.8°C, respectively, which correspond to the melting temperatures of these proteins in the absence of added solutes, while porcine liver esterase showed two thermal transitions at 61.8 and 83.6°C. This esterase occurs in solution in equilibrium between its monomeric and oligomeric forms (Dudman and Zerner 1975); hence, the first transition was attributed to the dissociation of the oligomeric protein and the second one to the melting of the monomer.

The trisaccharide produced a clear rise in all thermal transitions of the four tested enzymes (Table 2), performing slightly better than trehalose at the same molar concentration. However, the trisaccharide has a molar mass 1.5-fold greater than that of trehalose. Taking into account that in this range of concentrations the effect of protecting solutes on the melting temperature is fairly linear with solute concentration (Faria et al. 2008), the two solutes display similar relative abilities to stabilise proteins if we consider mass/volume concentration units.

Effect of gluconeotrehalose on the protection of enzymes against heat inactivation

The ability of gluconeotrehalose to protect enzyme activity from heat was assessed using four model enzymes: liver esterase, SNase, lysozyme and MDH. When esterase was incubated at 70°C for time periods up to 20 min, the residual activity in the absence of added solutes was always below 5% of the initial activity; remarkably, the addition of 0.5 M gluconeotrehalose allowed retaining ca. 60, 50, and 20% of the initial activity for incubation periods of 5, 10, and 20 min, respectively, while 0.5 M trehalose exerted no protection.

The incubation of staphylococcal nuclease at 99°C for 10, 20, and 30 min caused a progressive reduction in the activity of the enzyme to approximately 30% of the initial value. Trehalose or gluconeotrehalose (0.5 M concentration) exerted similar, but small, degrees of protection.

The effect on lysozyme protection was more irregular. At 80°C, trehalose was able to protect lysozyme against heat inactivation, increasing the residual activity after 10, 20, or 30 min of incubation by at least 30% in comparison with no solutes. The addition of gluconeotrehalose produced very similar results after 10 and 20 min of incubation; however, an incubation of 30 min abolished the

Table 2 Effect of trehalose and gluconeotrehalose at 0.5 M concentration on the melting temperature of staphylococcal nuclease (SNase), malate dehydrogenase (MDH), hen egg white lysozyme, and porcine liver esterase

	Increment of the melting temperature (°C)				
	SNase	MDH	Lysozyme	Esterase 1	Esterase 2
Trehalose	3.9 ± 0.9	3.4 ± 0.1	3.0	1.9 ± 0.3	2.1 ± 0.2
Gluconeotrehalose	5.4 ± 0.1	5.4 ± 0.6	4.7 ± 0.1	3.7 ± 0.3	3.0 ± 0.4

Esterase 1 and Esterase 2 refer, respectively, to the first and second thermal transitions observed with esterase. Esterase 2 is ascribed to melting of the monomer

residual activity of lysozyme. A similar behaviour was observed at 90°C, with trehalose being able to protect the enzyme up to 30 min of incubation, while gluconeotrehalose was very effective for 10 min of incubation, but totally ineffective after 20 or 30 min of incubation time.

Surprisingly, the addition of trehalose or gluconeotrehalose at 0.5 M stimulated the inactivation of MDH. In the absence of added solutes, a half-life for thermal inactivation of 4.4 ± 0.8 min was measured, while the addition of trehalose or gluconeotrehalose lowered this value to 3.7 ± 0.2 and 1.3 ± 0.5 min, respectively. In summary, except for the esterase which was highly stabilised by gluconeotrehalose, our results show that this unusual solute is not better than trehalose in the protection of enzymes against heat inactivation.

Discussion

In respect to NaCl requisites for growth, the bacterium *Carnobacterium* strain 17-4 shows typical halotolerant behaviour, displaying higher specific growth rates in the absence of added NaCl; however, the ability of this marine bacterium to grow at relatively high salt concentration (up to 10%) is noteworthy. Its categorisation in terms of temperature is more complicated. According to the strict Morita's (1975) definition, the optimal growth rate at 30°C places this isolate among the mesophiles; however, the maximal cell densities achieved between the growth temperatures of 10 and 15°C (at all salinities examined) and the poor growth yields above 25°C suggest a classification within the psychrotolerant organisms (Pikuta et al. 2007). Regardless of the classification, whose terms are still a matter of debate (Morita 1975; D'Amico et al. 2006; Pikuta et al. 2007), this is an organism that thrives efficiently at low temperatures and has the ability to cope with a relatively wide range of salinities, meaning that it is an interesting subject to study solute accumulation in cold environments.

The profile of solute accumulation by *Carnobacterium* strain 17-4 as a function of the salinity of the growth medium shows that the levels of gluconeotrehalose remained fairly constant throughout the whole range of salinity examined, with glycine-betaine appearing to serve as the main osmolyte under salt stress conditions (Fig. 4). Interestingly, in the absence of added salts, an increase in the growth temperature caused glycine-betaine to disappear from the solute pool, while the level of gluconeotrehalose was not significantly affected (Fig. 5). Therefore, these results provide no clue for the involvement of this rare trisaccharide in either heat protection or osmoprotection.

Gluconeotrehalose was able to increase the melting temperature of all the tested proteins to a level which surpassed that of trehalose at the same molar concentration, but if the observed increments are normalised taking into account the number of glucose units in gluconeotrehalose and trehalose, then we obtain similar stabilisation efficiency for the two solutes. Moreover, the performance of gluconeotrehalose as a protector of enzyme activity revealed to be generally feebler and very sensitive to the specific enzyme under study. This solute/protein specificity of the stabilising effect has been reported in the literature (Lippert and Galinski 1992; Lamosa et al. 2000; Borges et al. 2002), but in this case, it seems highly variable with examples of a protective ability above that of trehalose and examples of clear destabilisation.

These findings, combined with the fact that large amounts of gluconeotrehalose accumulated under all conditions examined (0.35 mg per mg of cell protein), might suggest a role as carbon reserve. However, the observation that the intracellular content of this trisaccharide was maintained in starved cells argues against that hypothesis. In view of the low temperature at the isolation site, the fjord of Spitsbergen, it would be interesting to test the efficacy of this solute in the protection of cold-sensitive proteins. At this stage, it is not possible to ascribe a physiological role to gluconeotrehalose in *Carnobacterium* strain 17-4.

The structure of this trisaccharide is especially interesting. Common trehalose (α,α configuration) is abundant in nature as a storage compound or stress protector in bacteria, archaea and fungi; in contrast, α,β-trehalose (also denominated neotrehalose) is extremely rare. Its occurrence has been described in honey, but its biosynthetic

pathway is unknown along with the organisms that are able to produce it. As a consequence, the properties of neotrehalose are poorly studied. The trisaccharide found in *Carnobacterium* strain 17-4 is the only derivative of neotrehalose reported so far, and to our knowledge, it has been found only in *Streptococcus faecalis* where its function remains elusive (Fisher and Krieglstein 1966). Nevertheless, tri- and tetra-saccharides derived from α,α-trehalose, such as α-glucosyl-(1→6)-α,α,-trehalose, α-glucosyl-(1→4)-α,α-trehalose, β-glucosyl-(1→6)-α,α-trehalose, or β-glucosyl-(1→3)-α,α-trehalose, have been found in insects, fungi, and bacteria serving as storage compounds or as part of membrane glycolipids (Esch et al. 1999; Hendrix and Salvucci 2001; Carbonero et al. 2001; Ohta et al. 2002; Biniukov et al. 1989).

Neotrehalose and its non-reducing glycosylated derivatives are expected to have interesting applications due to their high acid and thermal stability. However, the very low yields of chemical synthesis of neotrehalose and its low abundance in nature (below 1% in honey) (Swallow and Low 1990; Sanz et al. 2004) have encouraged the development of biochemical methods for its production (Yoshikatsu et al. 1996). In this context, *Carnobacterium* strain 17-4 could be an advantageous source for the production of this stable and rare disaccharide. This promise, along with the scarce distribution of gluconeotrehalose, raises great curiosity about the yet unknown biosynthetic enzymes and its unexplored biotechnological applications.

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