Antonie van Leeuwenhoek (2011) 99:681–695
DOI 10.1007/s10482-010-9544-z

ORIGINAL PAPER

Factors affecting accumulation and degradation of curdlan, trehalose and glycogen in cultures of *Cellulomonas flavigena* strain KU (ATCC 53703)

Lakmal S. Siriwardana · Aaron R. Gall · Clarence S. Buller · Steven W. Esch · William J. Kenyon

Received: 28 July 2010 / Accepted: 10 December 2010 / Published online: 29 December 2010

Abstract *Cellulomonas flavigena* strain KU (ATCC 53703) is a cellulolytic, Gram-positive bacterium which produces large quantities of an insoluble exopolysaccharide (EPS) when grown in minimal media with a high carbon-to-nitrogen (C/N) ratio. Earlier studies proved the EPS is structurally identical to the linear β-1,3-glucan known as curdlan and provided evidence that the EPS functions as a carbon and energy reserve compound. We now report that *C. flavigena* KU also accumulates two intracellular, glucose-storage carbohydrates under conditions of carbon and energy excess. These carbohydrates were partially purified and identified as the disaccharide trehalose and a glycogen/amylopectin-type polysaccharide. A novel method is described for the sequential fractionation and quantitative determination of all three carbohydrates from culture samples. This fractionation protocol was used to examine the effects of C/N ratio and osmolarity on the accumulation of cellular carbohydrates in batch culture. Increasing the C/N of the growth medium caused a significant accumulation of curdlan and glycogen but had a relatively minor effect on accumulation of trehalose. In contrast, trehalose levels increased in response to increasing osmolarity, while curdlan levels declined and glycogen levels were generally unaffected. During starvation for an exogenous source of carbon and energy, only curdlan and glycogen showed substantial degradation within the first 24 h. These results support the conclusion that extracellular curdlan and intracellular glycogen can both serve as short-term reserve compounds for *C. flavigena* KU and that trehalose appears to accumulate as a compatible solute in response to osmotic stress.

Keywords Cellulose · Disaccharide · Energy reserve · Glucan · Polysaccharide · Starvation

L. S. Siriwardana · A. R. Gall · W. J. Kenyon (✉)
Department of Biology, University of West Georgia, Carrollton, GA 30118, USA
e-mail: wkenyon@westga.edu

C. S. Buller · S. W. Esch
Department of Molecular Biosciences, The University of Kansas, Lawrence, KS 66045, USA

Present Address:
S. W. Esch
Division of Biology, Kansas Lipidomics Research Center, Kansas State University, Manhattan, KS 66506, USA

Introduction

The genus *Cellulomonas* includes nearly 20 species of coryneform bacteria known for their ability to degrade cellulose (Stackebrandt and Keddie 1984; Holt et al. 2000). This and other useful characteristics of *Cellulomonas* spp. make them attractive for a variety of potential applications in biotechnology

ranging from the production of single-cell protein and biofuels to bioremediation (Hitchner and Leatherwood 1980; McCool et al. 2000; Doi 2008; Viamajala et al. 2008; Wang and Chen 2009). The cellulomonads are described as Gram-positive, asporogenous, irregular rods which grow best aerobically often forming yellow colonies on solid media. They grow well at neutral pH and 30°C in complex media or in mineral-based media containing a suitable carbon and energy source and an ammonium salt as the nitrogen source. They are auxotrophic for biotin and/or thiamine, and these vitamins must either be added directly to minimal media or supplied in the form of a small amount of yeast extract.

Cellulomonas spp. have often been isolated from environmental samples using their ability to grow on cellulose as a selective factor. Using a cellulose enrichment technique, Angelo et al. (1990) isolated a new strain of *Cellulomonas flavigena* designated *C. flavigena* strain KU (ATCC 53703). However, this new isolate exhibited characteristics inconsistent with type strain *C. flavigena* (ATCC 482) (Angelo et al. 1990). One prominent feature of the new isolate was formation of large cellular aggregates when grown in minimal media with a high carbon-to-nitrogen (C/N) source ratio (Angelo et al. 1990; Voepel and Buller 1990). Electron microscopy of such cultures showed the presence of a thick capsule surrounding aggregated cells, and chemical analysis of aggregates revealed a high carbohydrate content (Angelo et al. 1990; Voepel and Buller 1990). An exopolysaccharide (EPS) was extracted from aggregated cells with NaOH and precipitated with either HCl or acetic acid to form a hydrogel (Buller and Voepel 1990). This purified EPS had solubility properties and chemical characteristics similar to a linear, β-1,3-glucan known as curdlan (Buller and Voepel 1990). Further structural work confirmed that *C. flavigena* KU produces an extracellular curdlan-type polysaccharide when grown in media with a high carbon-to-nitrogen (C/N) ratio (Kenyon and Buller 2002). Studies by Voepel and Buller (1990) clearly demonstrated that the curdlan EPS can be catabolized by *C. flavigena* KU if no other source of carbon and energy is available, and therefore, appears to function as an extracellular reserve compound. More recent studies have demonstrated that curdlan EPS is produced during growth of *C. flavigena* KU on a variety of substrates including cellulose and that the EPS forms the basis of an extracellular matrix promoting biofilm formation (Kenyon et al. 2005).

A series of earlier investigations reported that a closely related species known as *Cellulomonas uda* (ATCC 21399) accumulates two intracellular reserve carbohydrates identified as the disaccharide trehalose and a glycogen/amylopectin-type polysaccharide (Schimz et al. 1985; Schimz and Overhoff 1987a, b). Intriguingly, *C. uda* (ATCC 21399) is one of only a few *Cellulomonas* strains capable of producing large quantities of curdlan EPS (Buller and Voepel 1990; Kenyon et al. 2005). However, perhaps because of the high nitrogen content of their growth medium (Schimz et al. 1983), Schimz and Overhoff did not observe any cellular aggregation indicative of curdlan biosynthesis. These and other similarities between *C. flavigena* KU and *C. uda* (ATCC 21399) raised the question of whether *C. flavigena* KU is capable of producing trehalose and glycogen in addition to curdlan, and if so, which of these glucose-storage carbohydrates fulfill the criteria proposed by Wilkinson for a bacterial reserve compound (Wilkinson 1959; Dawes and Senior 1973).

Materials and methods

Microorganism, growth media, and culture conditions

Cellulomonas flavigena (ATCC 53703), known as strain KU (Angelo et al. 1990), was grown in either M9 minimal medium (Atlas 1993; Eisenstadt et al. 1994) or the nutritionally complete MOPS-salts minimal medium described by Neidhardt et al. (1974) supplemented with 0.05% yeast extract (i.e., M9YE or MSYE media). Standard media formulations were modified by changing the type of carbon source, carbon source concentration, NH_4Cl concentration, or NaCl concentration. Stock cultures were maintained on peptone-tryptone-yeast extract (PTYE) agar (Vladut-Taylor et al. 1986; Atlas 1993) or tryptic soy agar (Difco). All cultures were grown in baffled flasks and incubated aerobically at 30°C with continuous shaking at 200 rpm. Starter cultures were grown in standard minimal media for 24–48 h before being used to inoculate experimental cultures at a ratio of 1:100 (v:v).

Preparation of high-carbohydrate biomass

Cellulomonas flavigena KU high-carbohydrate biomass was prepared for testing different carbohydrate fractionation methods and as a source of material for the extraction, partial purification, and identification of trehalose, curdlan, and glycogen. High-carbohydrate biomass was prepared by first growing cultures in M9YE media containing a high carbon-to-nitrogen (C/N) ratio, 15 g l^{-1} (83.3 mM) glucose and 0.5 g l^{-1} (9.4 mM) NH_4Cl, for 3 days. Culture biomass was collected by centrifugation (10,800×*g* at 4°C for 10 min), washed by resuspension in cold physiological saline (150 mM NaCl at 4°C), and pelleted again. A second wash was performed with cold 15 mM NaCl, a concentration that prevents osmotic lysis but does not leave a significant mass of NaCl in the pellet (Herbert et al. 1971). Washed biomass was then lyophilized, ground to a fine powder, and stored over desiccant. This type of preparation routinely contained more than 90% carbohydrate by weight as determined by the phenol–sulfuric acid assay for total carbohydrate (Dubois et al. 1956).

Extraction and partial purification of trehalose, curdlan, and glycogen

Trehalose was extracted from high-carbohydrate biomass using a procedure based on the one described by Richtmyer (1962) for the isolation of α,α-trehalose from baker's yeast. A thick paste was prepared by mixing 10 g of high-carbohydrate biomass with 20 ml of distilled water. Seventy-five ml of 95% ethanol was added to the paste, and the resulting suspension was stirred for 30 min at room temperature. The suspension was then filtered through a glass microfiber filter (Whatman GF/A). The filter cake was washed three times with 70% ethanol and stored at 4°C for later isolation of curdlan and glycogen. The filtrate (i.e., ethanol extract) was concentrated to approximately 10 ml using a rotary evaporator at 45°C. Deproteinization of the concentrated solution was accomplished by the addition of 6 ml of aqueous 20% $ZnSO_4$, followed by 2 µl of 1% phenolphthalein and enough 0.3 N $BaOH_2$ to turn the indicator pink. After the addition of 0.6 g of decolorizing carbon, the mixture was heated to 70°C while stirring and filtered through a pad of Celite filter aide. Finally, the solution was passed through a column of ion-retardation resin (BioRad). This deproteinized, deionized solution was used for the identification of trehalose.

Curdlan was extracted from the remaining filter cake following protocol adapted from that described by Buller and Voepel (1990). The filter cake was resuspended in 1 N NaOH at a ratio of 1:100 (w:v) and stirred at room temperature for 30 min. The suspension was centrifuged (14,500×*g* at 4°C for 20 min), and the NaOH extract was collected by decantation. The pellet was extracted a second time, the two extracts were pooled, and the combined extract was filtered through a glass microfiber filter. The filtrate was neutralized with an equal volume of 1 N acetic acid. The resulting hydrogel was collected by centrifugation (10,800×*g* at 4°C for 10 min) and washed several times by resuspension in distilled water. This preparation was lyophilized, ground to a fine powder, and stored over desiccant.

A glycogen-type polysaccharide was extracted from the remaining residue using a modification of the method described by Sutherland and Wilkinson (1971). Following curdlan extraction, the pellet was resuspended in 5 N NaOH at a ratio of 1:100 (w:v) and heated in a boiling water bath for 1 h. After brief cooling in a room temperature water bath, the suspension was centrifuged (10,800×*g* at 25°C for 10 min), and the extract was collected by decantation. The extract was filtered through a glass microfiber filter, and polysaccharide was precipitated from the filtrate by the addition of three volumes of 95% ethanol. The polysaccharide was then pelleted by centrifugation (5,900×*g* at 4°C for 10 min) and redissolved in a small volume of distilled water at 60°C. After another round of precipitation, the polysaccharide preparation was lyophilized, ground to a fine powder, and stored over desiccant.

Quantitative and qualitative colorimetric assays

Total carbohydrate was determined using either the phenol–sulfuric acid method of Dubois et al. (1956) as described by Daniels et al. (1994) or the more hexose-specific anthrone–sulfuric acid assay (Herbert et al. 1971). The glucose oxidase assay (Sigma) was used to specifically determine D-glucose. Benedict's test (Robyt and White 1987) was used as a qualitative test for reducing-sugar.

Total cellular protein was extracted from washed biomass by two extractions with 1 N NaOH at 90°C for 10 min. The two extracts were pooled, diluted to a known volume with NaOH, and assayed using the method of Lowry et al. (1951) as described by Daniels et al. (1994) and using bovine serum albumin, also heated to 90°C for 10 min in 1 N NaOH, as the standard protein.

Acid hydrolysis of carbohydrates

Trehalose preparations were hydrolyzed with 1 M trifluoroacetic acid (TFA) under argon in a sealed test tube at 100°C for 4 h. Open tubes were then placed in a 45°C water bath and TFA removed by evaporation under a stream of nitrogen gas. The hydrolysate was dissolved in distilled water for further analysis. Polysaccharide preparations were first partially hydrolyzed with 90% formic acid under argon in a sealed test tube at 100°C for 1 h. Formic acid was then removed by evaporation at 45°C under a stream of nitrogen, and hydrolysis was completed in TFA as described above for trehalose.

Thin-layer chromatography of carbohydrates

Thin-layer chromatography (TLC) of carbohydrates was performed using Whatman K-5, 150 Å, silica gel plates with a layer thickness of 250 μm. Mono-, di-, and oligosaccharides were separated using four ascents of acetonitrile:water (85:15, v:v) (Robyt and White 1987). Visualization of carbohydrates was accomplished by spraying plates with sulfuric acid–methanol (1:3, v:v) and heating for 5 min at 110°C to give brown to black spots. Chromatographic mobilities are reported as the distance traveled relative to glucose (R_{Glc}).

Fractionation of cellular carbohydrates using a modified yeast protocol

A procedure was developed by Trevelyan and Harrison (1956) for fractionation of cellular carbohydrates from baker's yeast (*Saccharomyces cerevisiae*), and was later summarized by Herbert et al. (1971). The method results in the following order of biochemical fractions from yeast cells: trehalose, RNA, alkali-soluble glycogen, mannan, acid-soluble glycogen, and cell wall β-1,3-glucan. The protocol was modified herein by omitting the steps for fractionation of RNA and mannan and was evaluated for its potential use in the fractionation of cellular carbohydrates from *C. flavigena* KU.

Twenty-five mg of dry, high-carbohydrate biomass was suspended in 5 ml of ice-cold, 0.5 M trichloroacetic acid (TCA). The suspension was kept on ice for 30 min with intermittent vortexing. The suspension was then centrifuged (14,500×*g* at 0°C for 15 min), and the extract was decanted into a graduated test tube. The pellet was washed by resuspension in 5 ml of distilled water followed by another round of centrifugation. The wash was decanted and pooled with the extract, and the combined solution was diluted to a known volume. This TCA extract represented the trehalose fraction. The pellet remaining after TCA extraction was resuspended in 5 ml of 0.25 M sodium carbonate. The suspension was heated in a boiling water bath for 45 min and centrifuged (14,500×*g* at 25°C for 15 min). In a similar manner, the extract and a 5 ml wash of the pellet were combined and brought up to a known volume. This Na_2CO_3 extract represented the alkali-soluble glycogen fraction. The pellet remaining after Na_2CO_3 extraction was then resuspended in 5 ml of 0.5 M perchloric acid. The suspension was heated in a boiling water bath for 30 min and centrifuged (14,500×*g* at 25°C for 15 min). Again, the extract and a wash of the pellet were combined and diluted to a known volume. This $HClO_4$ extract represented the acid-soluble glycogen fraction. Finally, the residue, representing the β-1,3-glucan (i.e., curdlan) fraction, was resuspended in a known volume of 2 N NaOH by vigorous vortexing. The carbohydrate content of each fraction was determined using the anthrone–sulfuric acid assay with glucose as the standard. Results are reported as glucose equivalents of carbohydrate. Specific carbohydrate levels are reported as mg of carbohydrate per mg of cellular protein.

Fractionation of cellular carbohydrates using the *Cellulomonas* protocol

The following procedure was also evaluated for fractionation of cellular carbohydrates produced by *C. flavigena* KU. A 25 mg portion of high-carbohydrate biomass was suspended in 1 ml of distilled water, 4 ml of 95% ethanol was added, and the sample was vortexed. The suspension was placed in a

70°C water bath for 10 min. After brief cooling in a RT water bath, the suspension was centrifuged (14,500×*g* at 25°C for 15 min). The extract was decanted into a graduated test tube, and the pellet was extracted again in the same way. The two extracts were pooled and diluted to a known volume. This ethanol extract represented the trehalose fraction. The pellet remaining after trehalose extraction was resuspended in 5 ml of 1 N NaOH, and this suspension was allowed to stand at RT for 10 min before centrifugation (14,500×*g* at 25°C for 15 min). In a similar manner, the extract was decanted, the pellet was extracted again, and the two extracts were pooled. This NaOH extract represented the curdlan fraction. Glycogen could be extracted from the remaining pellet with 5 N NaOH at 100°C for 1 h, but this caused loss of carbohydrate due to alkaline degradation. Following extraction of glycogen, the residual pellet contained virtually no carbohydrate. Therefore, extraction of glycogen was considered an unnecessary step, and the pellet remaining after curdlan extraction was simply resuspended in a known volume of 1 N NaOH for determination of glycogen. The carbohydrate content of each fraction was determined using the anthrone–sulfuric acid assay with glucose as the standard. Results are reported as glucose equivalents of carbohydrate. Specific carbohydrate levels are reported as mg of carbohydrate per mg of cellular protein.

Results

Isolation and identification of curdlan, trehalose, and glycogen from high-carbohydrate biomass

Previous investigations established that *Cellulomonas uda* (ATCC 21399) produces both the disaccharide trehalose and a glycogen/amylopectin-type polysaccharide and that these carbohydrates can both serve reserves of carbon and energy (Schimz et al. 1985; Schimz and Overhoff 1987a, b). Interestingly, cellular morphology and vitamin requirements (Angelo et al. 1990), as well as biochemical tests results using the dichotomous key proposed by McHan and Cox (1987), all seem to indicate that *C. uda* (ATCC 21399) and *C. flavigena* KU (ATCC 53703) are more closely related to each other than either strain is to the *Cellulomonas* type strain *C. flavigena* (ATCC 482). Because of the similarities between these two strains, we decided to investigate whether *C. flavigena* KU was capable of producing trehalose and glycogen in addition to curdlan and to determine if these three storage carbohydrates accumulate simultaneously under conditions of carbon and energy excess.

Exopolysaccharide was extracted from *C. flavigena* KU high-carbohydrate biomass with 1 N NaOH and precipitated upon neutralization with acetic acid to form a viscous hydrogel as described by Buller and Voepel (1990). A detailed structural analysis of this type of polysaccharide preparation has been described elsewhere (Kenyon and Buller 2002) and provided evidence that this EPS is identical in structure to commercially available curdlan preparations (i.e., an unbranched β-1,3-glucan with a degree of polymerization of approximately 500 glucose residues).

To test for the presence of trehalose in *C. flavigena* KU, small molecular weight compounds were extracted from high-carbohydrate biomass with cold 0.5 M TCA. This type of extract contained a significant amount of anthrone-positive material, indicating the presence of hexoses. Cold perchloric acid, hot ethanol, and hot water extracts, performed as described by Herbert et al. (1971), also yielded anthrone-positive material. A modified version of the method described by Richtmyer (1962) was used to obtain a more purified preparation. The deproteinized and deionized extract resulting from this procedure also contained an anthrone-positive compound, and when this solution was tested for reducing sugars using Benedict's reagent (Robyt and White 1987), it gave a negative result. When the same solution was subjected to TLC, a single spot with an R_{Glc} value identical to authentic trehalose was visible. Furthermore, when the preparation was subjected to acid hydrolysis in 1 M TFA at 100°C for 2 h and then analyzed by TLC, a spot corresponding to glucose and a much fainter spot corresponding to trehalose were obtained. These results are summarized in Table 1 and are consistent with the biochemical properties of the non-reducing, glucose disaccharide trehalose.

When the modified Pflüger method for glycogen extraction described by Herbert et al. (1971) was used on *C. flavigena* KU high-carbohydrate biomass (Herbert et al. 1971; Sutherland and Wilkinson 1971), an anthrone-positive polysaccharide was obtained

Table 1 Summary of results supporting identification of trehalose and glycogen isolated from *C. flavigena* KU high-carbohydrate biomass

Trehalose preparation	Glycogen preparation
1. Positive anthrone reaction	1. Positive anthrone reaction
2. Water-soluble	2. Water-soluble
3. Negative Benedict's test	3. Insoluble in ≥60% ethanol
4. TLC gives R_{Glc} = authentic trehalose	4. TLC of hydrolysate gives R_{Glc} = glucose with trailing spots
5. TLC of hydrolysate gives R_{Glc} = glucose	5. Amyloglucosidase releases glucose

which was completely soluble in water and insoluble in 60% or greater ethanol. When digested with amyloglucosidase (Sigma), free glucose was detected with the glucose oxidase assay (Sigma). After hydrolysis of the polysaccharide in 1 M TFA at 100°C for 2 h, TLC revealed a spot corresponding to glucose and an unresolved series of oligosaccharides arising from incomplete hydrolysis with R_{Glc} values matching those of rabbit liver glycogen. These results (Table 1) are consistent with the biochemical properties of a glycogen/amylopectin-type polysaccharide.

Trehalose and total polysaccharide production from different carbon sources

In order to investigate whether the type of growth substrate has an affect on accumulation of *C. flavigena* KU carbohydrates, three cultures were prepared in M9YE media containing either glycerol, glucose, or cellobiose as the carbon source at a concentration of 1% and incubated aerobically at 30°C. At 24, 48, and 72 h of incubation, culture samples were removed, pelleted by centrifugation (14,500×*g* at 4°C for 10 min), and washed twice in physiological saline. Trehalose was extracted from washed cell pellets with cold TCA, and the remaining pellets were resuspended in distilled water by vigorous vortexing for the determination of total polysaccharide (i.e., curdlan plus glycogen). The amount of total carbohydrate in these two fractions was determined using the anthrone–sulfuric acid assay. Separate culture samples were taken for the extraction and determination of cellular protein. Specific carbohydrate levels are reported as mg carbohydrate per mg protein.

As shown in Table 2, the type of carbon source supplied to each culture did not have a significant impact on the amount of trehalose produced. However, relative to total cellular protein, cultures

Table 2 Trehalose and total polysaccharide production from different carbon sources

Carbon source	Incubation time (days)	Trehalose produced (mg carbohydrate mg protein^{-1})	Total polysaccharide produced (mg carbohydrate mg protein^{-1})
Glycerol	1	0.368 ± 0.050	0.568 ± 0.204
	2	0.838 ± 0.006	8.50 ± 0.11
	3	0.836 ± 0.037	12.3 ± 0.4
Glucose	1	0.644 ± 0.076	1.90 ± 0.15
	2	0.960 ± 0.013	16.0 ± 2.7
	3	0.919 ± 0.064	25.2 ± 5.9
Cellobiose	1	0.483 ± 0.030	3.25 ± 0.21
	2	0.655 ± 0.071	12.8 ± 0.2
	3	0.711 ± 0.025	20.0 ± 0.6

Cultures of *C. flavigena* KU were grown in M9YE media containing either glycerol, glucose, or cellobiose as the carbon and energy source at a concentration of 10.0 g l^{-1} (1.0%; w:v) and containing 0.125 g l^{-1} (2.5 mM) NH_4Cl. Each culture was incubated aerobically at 30°C for 72 h. Duplicate culture samples were removed every 24 h for the determination of trehalose and total polysaccharide and for determination of total cellular protein. Results are reported as mg carbohydrate per mg of total cellular protein. The data shown are sample averages from a representative experiment ± the standard deviation

supplied with either glucose or cellobiose accumulated more total polysaccharide compared to cultures grown on glycerol. Cultures grown on glucose produced approximately twice as much total polysaccharide compared to cultures grown on glycerol. Differences in curdlan production were clearly visible by an increase in cellular aggregation seen in cultures growing on glucose or cellobiose. Curdlan, trehalose, and glycogen were also produced during growth on cellulose and xylan, but unfortunately, the presence of these insoluble substrates interfered with the determination of total polysaccharide (data not shown).

Development of a carbohydrate fractionation protocol

In order to study the effects of culture conditions on the accumulation and degradation of *C. flavigena* KU carbohydrates, a practical method was needed for separating each carbohydrate from culture samples. The method developed by Trevelyan and Harrison (1956) for baker's yeast results in the following fractions: trehalose, total RNA, alkali-soluble glycogen, acid-soluble glycogen, mannan, and cell wall β-1,3-glucan. The total hexose content of each fraction is then determined by the anthrone–sulfuric acid assay, while RNA is determined by the orcinol–sulfuric acid assay. Because of similarities between some of the yeast and *Cellulomonas* carbohydrates, a modified version of this method, omitting steps for RNA and mannan, was tested on *C. flavigena* KU high-carbohydrate biomass. Results are shown in Table 3.

For comparison, an alternative carbohydrate fractionation procedure was developed specifically for *C. flavigena* KU biomass (Table 3). This simpler protocol, referred to as the "*Cellulomonas*" protocol, results in a trehalose fraction, a curdlan fraction, and a glycogen fraction. The total carbohydrate content of each fraction was determined using the anthrone–sulfuric acid assay. During development of this method, the effectiveness of extracting trehalose with 75% ethanol at 70°C and of extracting curdlan with 1 N NaOH at room temperature was examined. Two ethanol extractions were enough to remove most of the trehalose (99.3%). However, some carbohydrate, most likely curdlan, was present in a third NaOH extract. If only two NaOH extractions were performed, the small amount of remaining curdlan caused only a minor increase in the carbohydrate content of the final glycogen fraction. Therefore, two NaOH extractions were deemed sufficient for most purposes. The total amount of carbohydrate obtained from adding the three fractions was somewhat less than that obtained from whole biomass (Table 3). This was also true for the yeast protocol and can probably be attributed to the loss of some particulate matter during decantation of extracts. Because these fractionation methods were only used to get an estimate of carbohydrate levels, this was not considered a significant problem. The yield of trehalose using either protocol was nearly the same, with the yeast protocol yielding slightly less trehalose than the *Cellulomonas* protocol. The total amount of glycogen obtained from the yeast protocol (alkali-soluble plus acid-soluble) was significantly less than the value obtained in the *Cellulomonas* protocol. This was probably due to the thick curdlan capsule interfering with glycogen extraction. When non-aggregated cells (i.e., cells with no curdlan EPS) were used, values obtained for glycogen were similar using either protocol (Kenyon 1996). Furthermore, the value for curdlan was higher using the yeast protocol, and it follows that this was due to the presence of

Table 3 Evaluation of two methods for the fractionation of cellular carbohydrates

Carbohydrate fractions	mg carbohydrate
Modified yeast protocol	
1. Cold TCA (trehalose)	1.4
2. Hot Na_2CO_3 (alkali–glycogen)	0.1
3. Hot $HClO_4$ (acid–glycogen)	2.7
4. NaOH suspension (curdlan)	17.3
Total carbohydrate determined	21.5
Cellulomonas protocol	
1. Hot ethanol (trehalose)	1.4
2. RT NaOH (curdlan)	15.4
3. NaOH suspension (glycogen)	5.0
Total carbohydrate determined	21.8

Twenty-five mg of *C. flavigena* KU high-carbohydrate biomass (94% carbohydrate by weight) was fractionated as described in "Materials and methods" section and total carbohydrate was determined using the anthrone–sulfuric acid assay. Results are reported as mg carbohydrate (glucose equivalents) in each fraction and are average values obtained from duplicate 25 mg portions of dry biomass

unextracted glycogen in the final β-1,3-glucan (curdlan) fraction. It was clear from these results that the newly developed *Cellulomonas* protocol is a more efficient procedure for fractionation of carbohydrates from curdlan encapsulated cells. Therefore, the new *Cellulomonas* protocol was used in all subsequent experiments.

Effects of C/N on the accumulation of cellular carbohydrates

The carbon-to-nitrogen (C/N) ratio of growth media is known to induce the biosynthesis of bacterial carbon and energy reserves such as glycogen and poly-β-hydroxybutyrate (Dawes and Senior 1973). Because each of the storage carbohydrates produced by *C. flavigena* KU could potentially play a role in reserve metabolism, the following experiment was performed. Four cultures were prepared in MSYE media containing 10.0 g l^{-1} (55.5 mM) glucose and different concentrations of NH_4Cl as shown in Table 4. The cultures were incubated aerobically at 30°C for 2 days. Culture samples were removed at the end of incubation and washed twice in physiological saline. Trehalose, curdlan, and glycogen levels were determined using the *Cellulomonas* carbohydrate fractionation protocol. Separate culture samples were taken for extraction and determination of total cellular protein. Specific carbohydrate levels are reported as mg carbohydrate per mg protein.

Relative to total cellular protein, the amount of curdlan increased by a factor of 2.6-fold as the C/N ratio of the growth medium was raised by decreasing the NH_4Cl concentration from 10.0 to 1.25 mM. In comparison, both trehalose and glycogen levels increased by a factor of 1.8-fold (Table 4). Overall, glycogen levels were lower than curdlan levels, especially at higher C/N ratios. An increase in curdlan accumulation was also apparent, both macroscopically and microscopically, by a corresponding increase in cellular aggregation.

In a second set of experiments, the C/N ratio of the growth medium was increased by keeping the NH_4Cl concentration constant and raising the concentration of carbon source supplied to each culture. Four cultures were prepared in MSYE media containing 10 mM (0.5 g l^{-1}) NH_4Cl and varying concentrations of glucose as shown in Table 5. Cultures were incubated aerobically at 30°C for 2 days. Samples were removed at the end of incubation and washed twice in physiological saline. Trehalose, curdlan, and glycogen levels were determined using the *Cellulomonas* carbohydrate fractionation protocol. Separate culture samples were taken for extraction and determination of total cellular protein. Specific carbohydrate levels are reported as mg carbohydrate per mg protein. Results shown in Table 5 reveal a 2.0-fold increase in trehalose, a 6.7-fold increase in curdlan, and a 12.2-fold increase in glycogen when comparing cultures supplied with 0.5 and 2.0% glucose.

Based on the data presented in Tables 4 and 5, it is clear that accumulation of all three storage carbohydrates is affected by the C/N ratio of the growth medium. However, curdlan and glycogen levels appear to be influenced more than trehalose levels.

Effects of osmolarity on the accumulation of cellular carbohydrates

Trehalose has been shown to function in the osmoregulatory processes of many organisms ranging from bacteria to fungi to higher plants and animals. The

Table 4 Effects of nitrogen supply on the accumulation of cellular carbohydrates

Ammonium chloride (mM)	Cellular carbohydrates (mg carbohydrate mg protein^{-1})		
	Trehalose	Curdlan	Glycogen
10.0	0.469 ± 0.124	0.798 ± 0.137	0.470 ± 0.083
5.0	0.341 ± 0.065	1.04 ± 0.17	0.490 ± 0.101
2.5	0.679 ± 0.208	1.93 ± 0.30	0.529 ± 0.089
1.25	0.832 ± 0.177	2.09 ± 0.31	0.869 ± 0.135

Cultures of *C. flavigena* KU were grown aerobically in MSYE media containing 10.0 g l^{-1} (55.5 mM) glucose and NH_4Cl concentrations shown at 30°C for 48 h. Culture samples were then removed for fractionation and determination of cellular carbohydrates and protein. Results are reported as mg carbohydrate per mg total cellular protein ± the standard error from the mean (SEM) determined from three independent experiments

Table 5 Effects of carbon supply on the accumulation of cellular carbohydrates

Glucose (g l^{-1})	Cellular carbohydrates (mg carbohydrate mg $protein^{-1}$)		
	Trehalose	Curdlan	Glycogen
5.0	0.363 ± 0.098	0.719 ± 0.101	0.176 ± 0.092
10.0	0.571 ± 0.176	2.99 ± 1.32	0.820 ± 0.313
15.0	0.653 ± 0.150	3.42 ± 0.91	0.599 ± 0.604
20.0	0.714 ± 0.163	4.81 ± 1.44	2.14 ± 0.77

Cultures of *C. flavigena* KU were grown aerobically in MSYE media with varying concentrations of glucose and 0.5 g l^{-1} (10 mM) NH_4Cl at 30°C for 48 h. Culture samples were then removed for fractionation and determination of cellular carbohydrates and protein. Results are reported as mg carbohydrate per mg total cellular protein ± the standard error from the mean (SEM) determined from three independent experiments

following experiment was performed to determine if osmotic stress influences the amount of trehalose produced by *C. flavigena* KU. Four cultures were prepared in MSYE media containing 10.0 g l^{-1} (55.5 mM) glucose, 0.5 g l^{-1} (10 mM) NH_4Cl, and increasing concentrations of NaCl as shown in Table 6. Cultures were incubated aerobically at 30°C for 2 days. At the end of incubation, culture samples were removed, washed twice in cold physiological saline, and cellular carbohydrates determined using the *Cellulomonas* carbohydrate fractionation protocol. Separate samples were taken for extraction and determination of total cellular protein. Specific carbohydrate levels are reported as mg carbohydrate per mg protein.

As the NaCl concentration of the growth medium was raised from 50 mM (2.9 g l^{-1}) to 400 mM (23.4 g l^{-1}), there was a corresponding 2.4-fold increase in the amount of trehalose relative to total cellular protein (Table 6). Within the same range of NaCl concentrations, curdlan levels decreased steadily, dropping 1.7-fold, and glycogen levels remained essentially unchanged. Very little macroscopic aggregation was visible in high-salt cultures, also indicating a limited accumulation of curdlan EPS. Furthermore, increased trehalose synthesis was detected in cultures supplied with more than 5% glucose (data not shown). Taken together, these data suggest that trehalose accumulates as a compatible solute in *C. flavigena* KU under conditions of hyperosmotic stress.

Degradation of cellular carbohydrates during carbon and energy starvation

The following experiment was performed to investigate the degradation rates of trehalose, curdlan, and glycogen during starvation for an exogenous source of carbon and energy. A culture of *C. flavigena* KU was grown aerobically at 30°C for 3 days in M9YE medium containing 16 g l^{-1} of glucose (88.8 mM) and 0.25 g l^{-1} of NH_4Cl (4.7 mM). Samples of this culture were washed twice in M9YE medium lacking glucose. The washed biomass was resuspended in

Table 6 Effects of osmolarity on the accumulation of cellular carbohydrates

Sodium chloride (mM)	Cellular carbohydrates (mg carbohydrate mg $protein^{-1}$)		
	Trehalose	Curdlan	Glycogen
50	0.393 ± 0.091	3.07 ± 0.87	0.938 ± 0.284
100	0.765 ± 0.175	2.19 ± 0.46	0.918 ± 0.298
200	0.766 ± 0.152	2.05 ± 0.42	1.16 ± 0.33
400	0.927 ± 0.217	1.77 ± 0.50	0.942 ± 0.333

Cultures of *C. flavigena* KU were grown aerobically in MSYE media containing 10.0 g l^{-1} (55.5 mM) glucose, 0.5 g l^{-1} (10 mM) NH_4Cl, and NaCl concentrations shown at 30°C for 48 h. Culture samples were then removed for fractionation and determination of cellular carbohydrates and protein. Results are reported as mg carbohydrate per mg total cellular protein ± the standard error from the mean (SEM) determined from three independent experiments

fresh M9YE medium without glucose and transferred to a new culture flask. This starvation culture was incubated aerobically at 30°C, and samples were removed at regular time intervals for fractionation and determination of cellular carbohydrates using the *Cellulomonas* carbohydrate fractionation protocol. Separate culture samples were removed for extraction and determination of total cellular protein. Specific carbohydrate levels were reported as mg carbohydrate per mg protein.

During the first 24 h of starvation for an exogenous source of carbon and energy, trehalose levels actually increased 1.2-fold relative to total cellular protein, whereas curdlan and glycogen were degraded by a factor of 14.8- and 7.9-fold, respectively (Table 7). Earlier time points did not reveal any difference in the relative degradation rates of curdlan and glycogen, and later time points indicated no further significant decline in curdlan and glycogen levels detectable by our method (Table 7). Clearly, both curdlan and glycogen can be catabolized as short-term reserves if no other carbon and energy source is available. Trehalose levels did drop by 1.8-fold between 24 and 72 h of starvation (Table 7), perhaps indicating a long-term reserve function as proposed by Schimz and Overhoff (1987b).

Interestingly, cellular protein levels increased by a factor of 6.3-fold within the first 24 h of starvation and then remained fairly constant for the next 48 h (Table 7), suggesting that degradation of curdlan and glycogen was supporting at least some protein synthesis. Because of extensive cellular aggregation in such cultures, the number of colony-forming-units (CFUs) per ml could not be determined reliably, but an increase in CFUs was detectable within the first 24 h of starvation (data not shown). This was probably due to both a limited amount of growth and the extensive disaggregation observed by visual inspection of the culture, by examination of samples under phase-contrast microscopy, and by an increase in optical density (OD_{600}) following sedimentation of large aggregates.

Discussion

After the EPS produced by *C. flavigena* KU (ATCC 53703) was confirmed to be a linear β-1,3-glucan identical to curdlan (Kenyon and Buller 2002), the focus of our research turned to an investigation of its physiological function. To date, very little is known about the biological role of curdlan produced by other bacteria (McIntosh et al. 2005). Curdlan produced by certain strains of *Alcaligenes* or *Agrobacterium* is loosely associated with cells, and can often be found in the culture medium completely free from cells (Nakanishi et al. 1976). Some of these curdlan-producing strains also produce substantial amounts of anionic, water-soluble EPS. In contrast, the curdlan EPS produced by *C. flavigena* KU remains completely cell-associated, and no other EPSs have been detected (Buller and Voepel 1990; Kenyon and Buller 2002). Kenyon et al. (2005) provided evidence that the curdlan EPS produced by *C. flavigena* KU may form the basis of an extracellular biofilm matrix involved in cellulose degradation. In addition, there was already substantial proof that extracellular curdlan can serve as a source of carbon and energy for *C. flavigena* KU (Voepel and Buller 1990), whereas the curdlan produced by other strains of bacteria does

Table 7 Cellular carbohydrate and protein levels during starvation for an exogenous carbon and energy source

Starvation time (h)	Cellular carbohydrates (mg carbohydrate mg protein^{-1})			Cellular protein (mg protein ml culture^{-1})
	Trehalose	Curdlan	Glycogen	
T_0	0.585 ± 0.086	8.94 ± 1.34	5.53 ± 1.27	2.84 ± 0.21
T_{24}	0.710 ± 0.059	0.585 ± 0.090	0.688 ± 0.184	17.6 ± 1.3
T_{48}	0.340 ± 0.074	0.457 ± 0.114	0.594 ± 0.124	19.7 ± 1.7
T_{72}	0.409 ± 0.091	0.497 ± 0.050	0.747 ± 0.251	17.4 ± 0.8

C. flavigena KU was grown aerobically in M9YE media containing 15.0 g l^{-1} (83.3 mM) glucose and 0.25 g l^{-1} (4.7 mM) NH_4Cl at 30°C for 72 h. Biomass was collected by centrifugation and washed twice in sterile M9YE media with no glucose. Washed biomass was then resuspended in fresh M9YE media without glucose and incubated aerobically at 30°C for 72 h. Duplicate culture samples were removed at 24 h intervals for fractionation and determination of cellular carbohydrates and protein. The data shown are sample averages from a representative experiment ± the standard deviation

not appear to serve this function. However, it should be noted that long term cultures of *Alcaligenes faecalis* var. *myxogenes* IFO 13140 resulted in curdlan preparations with a lower degree of polymerization (DP) due to partial degradation (Nakanishi et al. 1974).

Voepel and Buller (1990) tentatively concluded that the curdlan EPS produced by *C. flavigena* KU functions as a carbon and energy reserve based on the following observations. Typical of a bacterial reserve compound, accumulation of curdlan EPS was promoted by growth of cultures in media with a high C/N ratio. When aggregated cells were incubated in media lacking an exogenous source of carbon and energy, cells disaggregated, the EPS capsule seen under electron microscopy disappeared, and cellular carbohydrate content declined. In addition, cultures of *C. flavigena* KU could be grown on purified EPS as the sole source of carbon and energy. Voepel (1989) also found that *C. flavigena* KU is capable of secreting both exo- and endo-β-1,3-glucanases. These enzymes are believed to be responsible for degradation of curdlan under starvation conditions.

It is unusual for any type of bacterial reserve compound to be accumulated extracellularly. This location would theoretically expose the compound to degradation by other microorganisms. However, curdlan EPS may be difficult for other organisms to digest because of its native physical structure, because it is associated with other capsular material, or simply because few microorganisms have enzymes capable of degrading β-1,3-glucans. In addition, it would seem to be advantageous for such an extracellular reserve to remain closely associated with cells that produce it and for it to be insoluble so as not to diffuse away from cells.

The two most common bacterial carbon and energy reserves are glycogen/amylopectin-type polysaccharides (α-1,4-glucans with a high number of α-1,6 branch points) and poly-β-hydroxybutyrate (PHB) (Dawes and Senior 1973). Glycogen-type polysaccharides are fairly common among coryneform bacteria such as *Arthrobacter* spp. (Zevenhuizen 1966, 1992) and the related actinomycetes such as *Mycobacterium* spp. (Elbein and Mitchell 1973), *Frankia* spp. (Lopez et al. 1984), and *Streptomyces* spp. (Braña et al. 1986). As discussed above, Schimz and Overhoff (1987a) discovered a glycogen-like polysaccharide, as well as the disaccharide trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), in extracts of *Cellulomonas uda* (DSM 20108, ATCC 21399), but were unaware that this strain also produces extracellular curdlan under certain growth conditions (Buller and Voepel 1990; Kenyon et al. 2005). Based on their studies, Schimz and Overhoff concluded that both storage carbohydrates acted as carbon and energy reserve compounds, but that trehalose degradation was responsible for maintenance of long-term viability (Schimz and Overhoff 1987b). In addition, these authors did not detect any PHB or polyphosphate from this strain. The polysaccharide isolated by Schimz and Overhoff (1987a) after either hot perchloric acid or hot alkali extraction of *C. uda* (ATCC 21399) was identified as a glycogen-type polysaccharide based on its digestion with amylases and effects on the absorption spectra of iodine/iodide solutions. However, some of their results indicated differences between this polysaccharide and authentic glycogen-type polysaccharides from other sources. Only 27% of the polysaccharide was converted to maltose by β-amylolysis. When ammonium sulfate was added to the glycogen–iodine/iodide solution, the absorption maximum decreased instead of increasing as it did with rabbit liver glycogen. If cells contained even small amounts of curdlan EPS, the glycogen-like polysaccharide could have possibly become contaminated with short, water-soluble, β-1,3-glucans generated during hot perchloric acid or hot alkali extraction. If these oligosaccharides were present, they may have contributed to these atypical results.

We report here that *C. flavigena* KU also produces trehalose and a glycogen/amylopectin-type polysaccharide in addition to curdlan EPS, and after the development of a novel method for the fractionation and quantitative determination of these cellular carbohydrates, the role of each carbohydrate in reserve metabolism was investigated. Wilkinson's first criterion for a bacterial energy reserve states, "The compound is accumulated under conditions when the supply of energy from exogenous sources is in excess of that required by the cell for growth and related processes at that particular moment of time." (Wilkinson 1959; Dawes and Senior 1973). This first criterion was examined both by supplying *C. flavigena* KU cultures with decreasing amounts of NH_4Cl and by supplying cultures with increasing amounts of glucose, and thus, increasing the C/N of the medium (Tables 4, 5). The results of these experiments

indicate that both curdlan and glycogen clearly meet this criterion, whereas the results for trehalose are less convincing. Similar results were obtained for trehalose and glycogen produced by *C. uda* (ATCC 21399) (Schimz and Overhoff 1987a).

Wilkinson's second criterion, "The compound is utilized when the supply of energy from exogenous sources is insufficient for the optimal maintenance of the cell, either for growth and division or for maintenance of viability and other processes" (Wilkinson 1959; Dawes and Senior 1973), was addressed by incubating cells with a high carbohydrate content in media lacking an exogenous carbon and energy source (Table 7). Curdlan and glycogen were simultaneously degraded within the first 24 h of starvation, whereas trehalose levels rose slightly during the same time period. These results were expected for curdlan based on the results obtained previously by Voepel and Buller (1990) and agree well with the observations of Schimz and Overhoff (1987b) who reported a relatively rapid decline for glycogen and a slower decline for trehalose during starvation of *C. uda* (ATCC 21399). Therefore, our results indicate that both the curdlan and glycogen polysaccharides produced by *C. flavigena* KU meet Wilkinson's second criterion for bacterial energy reserves.

The third criterion states, "The compound is broken down to produce energy in a form utilizable by the cell, and that it is, in fact, utilized for some purpose which gives the cell a biological advantage in the struggle for existence over those cells which do not have a comparable compound" (Wilkinson 1959; Dawes and Senior 1973). The degradation of curdlan and glycogen within the first 24 h of starvation supplied enough carbon and energy for a limited amount of growth as indicated by an increase in total cellular protein (Table 7). Thus, both curdlan and glycogen met the first part of this criterion, however, the second part of Wilkinson's third criterion could not be adequately addressed because of the lack of mutants unable to synthesize curdlan and glycogen. In conclusion, both polysaccharides (i.e., curdlan and glycogen) fulfilled the criteria proposed by Wilkinson (1959) for a bacterial energy reserve compound, whereas trehalose did not entirely behave as such. The reason why *C. flavigena* KU would require two different reserve polysaccharides remains unclear.

For several years, evidence has been building that supports a stress-protective role for trehalose. Trehalose has been shown to protect bacterial cells against matric and osmotic water stress as well as temperature stress (Van Laere 1989; Wiemken 1990; Elbein et al. 2003). In fact, the principal function of trehalose in prokaryotes may be to serve as a compatible solute which is accumulated in the cytosol during periods of hyperosmotic stress (Zevenhuizen 1992; Csonka 1989; Empadinhas and da Costa 2008). Because it has no free reducing-ends, trehalose does not react with other cellular constituents, making it an ideal compatible solute. By increasing the NaCl concentration of the growth medium, we have shown that trehalose accumulates in cells of *C. flavigena* KU in response to hyperosmotic stress (Table 6).

Trehalose is also thought to protect both vegetative cells and spores against the damaging effects of desiccation by binding to proteins and membrane lipids and, in the process, essentially replacing water (Crowe et al. 1992; Crowe 2007). In so doing, trehalose keeps proteins from denaturing and keeps the cell membrane in a fluid state. Interestingly, curdlan may also act to protect cells from desiccation stress because Okuyama et al. (1991) showed that curdlan holds onto bound water under conditions of very low relative humidity, and Farney (1993) demonstrated that the viability of encapsulated *C. flavigena* KU cells is maintained even after prolonged periods of dehydration. Therefore, curdlan and trehalose may both play a part in the desiccation tolerance of *C. flavigena* KU.

We also investigated the accumulation of trehalose and total polysaccharides during growth of *C. flavigena* KU on different carbon sources (Table 2). Our results indicate that the type of carbon source supplied to a culture influences accumulation of storage carbohydrates. A possible explanation for these findings involves the fact that *C. uda* (ATCC 21399) was shown to synthesize a cellobiose phosphorylase whose activity is localized in the soluble, cytosol fraction of cells (Schimz et al. 1983; Nidetzky et al. 2000). This type of enzyme (cellobiose:orthophosphate glucosyltransferase, EC 2.4.1.20) uses inorganic phosphate to phosphorolytically cleave cellobiose into one mole of α-D-glucose-1-phosphate and one mole of D-glucose. Part of the energy of the glycosidic bond of cellobiose is thus conserved in this

reaction. The glucose-1-phosphate can be directly activated to nucleotide sugar for polysaccharide synthesis, UDP-Glc for EPS synthesis or ADP-Glc for glycogen synthesis, or it can be converted into glucose-6-phosphate, by the action of phosphoglucomutase, and enter the EMP or pentose phosphate pathways to produce energy (Marschoun et al. 1987). Thus, by using cellobiose phosphorylase, one mole of ATP is saved in the phosphorylation of the two glucose moieties of cellobiose. Schimz et al. (1983) noted that bacteria with this enzyme grow better on cellobiose versus glucose, and the type of carbon and energy source supplied to cultures of *C. uda* (ATCC 21399) did make a considerable difference in the amount of trehalose (Schimz et al. 1985) and glycogen (Schimz and Overhoff 1987a) accumulated. Because *C. flavigena* KU and *C. uda* (ATCC 21399) appear to be closely related, it is likely that strain KU also possesses this enzyme. In support of this hypothesis, our results show that both glucose and cellobiose resulted in a more rapid accumulation of total polysaccharide compared to glycerol (Table 2). These results might be also be explained by a slower growth rate on glycerol due to the fact that once glycerol enters the cell, usually by facilitated diffusion, it has to be phosphorylated and converted to glucose-6-phosphate, via gluconeogenesis, before carbohydrate synthesis can proceed. In either case, polysaccharide synthesis from glycerol, or glycerol-yielding substrates, requires more energy than it does from either glucose or cellobiose.

In conclusion, we have shown that *C. flavigena* strain KU (ATCC 53703) simultaneously produces three glucose-storage carbohydrates during growth on a variety of substrates. In addition, we have devised a novel protocol for the sequential fractionation and quantitative determination of these cellular carbohydrates. Our results provide evidence supporting a short-term reserve function for curdlan and glycogen and an osmoprotective role for trehalose. Our findings are further supported by the fact that the complete genome sequence of *C. flavigena* type strain 134^T (ATCC 482, DSM 20109) contains several ORFs that appear to encode enzymes involved in the biosynthesis and catabolism of trehalose, glycogen, and exopolysaccharides (Abt et al. 2010). Based on these results, as well as results from earlier studies (Kenyon et al. 2005), *C. flavigena* KU appears to grow on cellulose as an adherent biofilm that converts the glucose residues of cellulose into three different storage carbohydrates; extracellular curdlan and intracellular trehalose and glycogen. In the process, very little soluble sugar is released into the surrounding environment (Kenyon et al. 2005). This mode of growth may have implications for the use of this organism in certain biotechnological applications including the industrial production of curdlan, single-cell protein, or biofuels.

Acknowledgments This work was supported by Columbian Resources grant 5363-XO-0705 (to C.S.B.), by funding from the University of West Georgia Student Research Assistantship Program (to W.J.K.), and by a Graduate Research Assistantship from the University of West Georgia (to L.S.S.). The authors would like to thank Dr. Sara J. Molesworth-Kenyon of the University of West Georgia for her help in the preparation of this manuscript.

References

Abt B, Foster B, Lapidus A, Clum A, Sun H, Pukall R, Lucas S, Del Rio T, Nolan M, Tice H, Cheng JF, Pitluck S, Liolios K, Ivanova N, Mavromatis K, Ovchinnikova G, Pati A, Goodwin L, Chen A, Palaniappan K, Land M, Hauser L, Chang YJ, Jeffries CD, Rohde M, Göker M, Woyke T, Bristow J, Eisen JA, Markowitz V, Hugenholtz P, Kyrpides N, Klenk HP (2010) Complete genome sequence of *Cellulomonas flavigena* type strain (134^T). Stand Genomic Sci 3:15–25

Angelo R, Voepel KC, Buller CS (1990) Isolation and characterization of a new strain of *Cellulomonas flavigena*. J Ind Microbiol 5:125–130

Atlas RM (1993) Handbook of microbiological media. CRC Press, Ann Arbor

Braña AF, Méndez C, Díaz LA, Manzanal MB, Hardisson C (1986) Glycogen and trehalose accumulation during colony development in *Streptomyces antibioticus*. J Gen Microbiol 132:1319–1326

Buller CS, Voepel KC (1990) Production and purification of an extracellular polyglucan produced by *Cellulomonas flavigena* strain KU. J Ind Microbiol 5:139–146

Crowe JH (2007) Trehalose as a "chemical chaperone": fact and fantasy. Adv Exp Med Biol 594:143–158

Crowe JH, Hoekstra FA, Crowe LM (1992) Anhydrobiosis. Annu Rev Physiol 54:579–599

Csonka LN (1989) Physiological and genetic responses of bacteria to osmotic stress. Microbiol Rev 53:121–147

Daniels L, Hanson RS, Phillips JA (1994) Chemical analysis. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC, pp 512–554

Dawes EA, Senior PJ (1973) The role and regulation of energy reserve polymers in micro-organisms. In: Rose AH, Tempest DW (eds) Advances in microbial physiology, vol 10. Academic Press, New York, pp 136–266

Doi RH (2008) Cellulases of mesophilic microorganisms: cellulosome and noncellulosome producers. Ann NY Acad Sci 1125:267–279

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for the determination of sugars and related substances. Anal Chem 28:350–356

Eisenstadt E, Carlton BC, Brown BJ (1994) Gene mutation. In: Gerhardt P, Murray GE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC, pp 297–316

Elbein AD, Mitchell M (1973) Levels of glycogen and trehalose in *Mycobacterium smegmatis* and the purification and properties of the glycogen synthetase. J Bacteriol 113: 863–873

Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multifunctional molecule. Glycobiology 13:17–27

Empadinhas N, da Costa MS (2008) Osmoadaptation mechanisms in prokaryotes: distribution of compatible solutes. Int Microbiol 11:151–161

Farney MS (1993) Structural and functional role of an extracellular polysaccharide produced by *Cellulomonas flavigena* strain KU. Thesis, The University of Kansas

Herbert D, Phipps PJ, Strange RE (1971) Chemical analysis of microbial cells. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 5B. Academic Press, New York, pp 209–344

Hitchner EV, Leatherwood JM (1980) Use of a cellulase-derepressed mutant of *Cellulomonas* in the production of a single-cell protein product from cellulose. Appl Environ Microbiol 39:382–386

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (2000) Genus *Cellulomonas*. In: Hensyl WR (ed) Bergey's manual of determinative bacteriology, 9th edn. Lippincott Williams & Wilkins, Philadelphia, p 575

Kenyon WJ (1996) Structure and function of a capsular polysaccharide from *Cellulomonas flavigena* strain KU. Dissertation, The University of Kansas

Kenyon WJ, Buller CS (2002) Structural analysis of the curdlan-like exopolysaccharide produced by *Cellulomonas flavigena* KU. J Ind Microbiol Biotechnol 29: 200–203

Kenyon WJ, Esch SW, Buller CS (2005) The curdlan-type exopolysaccharide produced by *Cellulomonas flavigena* KU forms part of an extracellular glycocalyx involved in cellulose degradation. Antonie van Leeuwenhoek 87: 143–148

Lopez MF, Fontaine MS, Torrey JG (1984) Levels of trehalose and glycogen in *Frankia* sp. HFPArl3 (Actinomycetales). Can J Microbiol 30:746–752

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

Marschoun S, Rapp P, Wagner F (1987) Metabolism of hexoses and pentoses by *Cellulomonas uda* under aerobic conditions and during fermentation. Can J Microbiol 33:1024–1031

McCool CS, Shaw AK, Singh A, Bhattacharya S, Green DW, Willhite GP (2000) Permeability reduction by treatment with KUSP1 biopolymer. SPE J 5:371–376

McHan F, Cox NA (1987) Differentiation of *Cellulomonas* species using biochemical tests. Lett Appl Microbiol 4:33–36

McIntosh M, Stone BA, Stanisich VA (2005) Curdlan and other bacterial (1 → 3)-β-D-glucans. Appl Microbiol Biotechnol 68:163–173

Nakanishi I, Kimura K, Kusui S, Yamazaki E (1974) Complex formation of gel-forming bacterial (1,3)-β-D-glucans (curdlan-type polysaccharides) with dyes in aqueous solution. Carbohydr Res 32:47–52

Nakanishi I, Kimura K, Suzuki T, Ishikawa M, Banno I, Sakane T, Harada T (1976) Demonstration of curdlan-type polysaccharide and some other β-1,3-glucan in microorganisms with aniline blue. J Gen Appl Microbiol 22:1–11

Neidhardt FC, Bloch PL, Smith DF (1974) Culture medium for enterobacteria. J Bacteriol 119:736–747

Nidetzky B, Eis C, Albert M (2000) Role of non-covalent enzyme-substrate interactions in the reaction catalysed by cellobiose phosphorylase from *Cellulomonas uda*. Biochem J 351:649–659

Okuyama K, Otsubo A, Fukuzawa Y, Ozawa M, Harada T, Kasai N (1991) Single-helical structure of native curdlan and its aggregation state. J Carbohydr Chem 10:645–656

Richtmyer NK (1962) α,α-Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) dihydrate: isolation from baker's yeast. In: Whistler RL, Wolfrom ML, BeMiller JN, Shafizadeh F (eds) Methods in carbohydrate chemistry, vol 1. Academic Press, New York, pp 370–372

Robyt JF, White BJ (1987) Chromatographic techniques. In: Biochemical techniques: theory and practice. Waveland Press, Prospect Heights, pp 73–128

Schimz KL, Overhoff B (1987a) Glycogen, a cytoplasmic reserve polysaccharide of *Cellulomonas* sp. (DSM20108): its identification, carbon source-dependent accumulation, and degradation during starvation. FEMS Microbiol Lett 40:325–331

Schimz KL, Overhoff B (1987b) Investigations of the influence of carbon starvation on the carbohydrate storage compounds (trehalose, glycogen), viability, adenylate pool, and adenylate energy charge in *Cellulomonas* sp. (DSM20108). FEMS Microbiol Lett 40:333–337

Schimz KL, Broll B, John B (1983) Cellobiose phosphorylase (EC 2.4.1.20) of *Cellulomonas*: occurrence, induction, and its role in cellobiose metabolism. Arch Microbiol 135: 241–249

Schimz KL, Irrgang K, Overhoff B (1985) Trehalose, a cytoplasmic reserve disaccharide of *Cellulomonas* sp. DSM20108: its identification, carbon source-dependent accumulation, and degradation during starvation. FEMS Microbiol Lett 30:165–169

Stackebrandt E, Keddie RM (1984) Cellulomonas. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology, vol 2. The Williams and Wilkins Co, Baltimore, pp 1325–1329

Sutherland IW, Wilkinson JF (1971) Chemical extraction methods in microbial cells. In: Norris JR, Ribbons DW (eds) Methods in microbiology, vol 5B. Academic Press, New York, pp 345–383

Trevelyan WE, Harrison JS (1956) Studies on yeast metabolism: yeast carbohydrate fractions: separation from

nucleic acid, analysis, and behavior during anaerobic fermentation. Biochem J 63:23–33

Van Laere A (1989) Trehalose, reserve and/or stress metabolite? FEMS Microbiol Rev 63:201–210

Viamajala S, Peyton BM, Gerlach R, Sivaswamy V, Apel WA, Petersen JN (2008) Permeable reactive biobarriers for in situ Cr(VI) reduction: bench scale tests using *Cellulomonas* sp. strain ES6. Biotechnol Bioeng 101:1150–1162

Vladut-Taylor M, Kauri T, Kushner DJ (1986) Effects of cellulose on growth, enzyme production, and ultrastructure of a *Cellulomonas* species. Arch Microbiol 144:191–195

Voepel KC (1989) Formation of an extracellular energy reserve by *Cellulomonas flavigena* strain KU. Dissertation, The University of Kansas

Voepel KC, Buller CS (1990) Formation of an extracellular energy reserve by *Cellulomonas flavigena* strain KU. J Ind Microbiol 5:131–138

Wang ZW, Chen S (2009) Potential of biofilm-based biofuel production. Appl Microbiol Biotechnol 83:1–18

Wiemken A (1990) Trehalose in yeast, stress protectant rather than reserve carbohydrate. Antonie van Leeuwenhoek 58:209–217

Wilkinson JF (1959) The problem of energy-storage compounds in bacteria. Exp Cell Res Suppl 7:111–130

Zevenhuizen LPTM (1966) Formation and function of a glycogen-like polysaccharide of *Arthrobacter*. Antonie van Leeuwenhoek 32:356–372

Zevenhuizen LPTM (1992) Levels of trehalose and glycogen in *Arthrobacter globiformis* under conditions of nutrient starvation and osmotic stress. Antonie van Leeuwenhoek 61:61–68