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RESEARCH ARTICLE

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Rapid extracellular acidification induced by glucose metabolism in non-proliferating cells of *Serratia marcescens*

Summary The addition of glucose or other sugars to resting cells of *Serratia marcescens* induced rapid acidification of the extracellular medium. This acidification was due to the catabolism of sugars. The rate of acidification depended on the carbon source and its concentration. HPLC analysis of the supernatants demonstrated that the progressive fall in pH resulted from the rapid production of lactic, acetic, pyruvic and citric acids. Other microorganisms were tested for their ability to produce this rapid acidification of the medium. This study may provide a rapid and simple method for metabolism studies.

Key words *Serratia marcescens* · *Enterococcus faecalis* · *Escherichia coli* · *Pseudomonas aeruginosa* · Extracellular acidification

Introduction

Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites [2–4, 6, 8, 10–13, 16]. Glucose, which is usually an excellent carbon source for bacterial growth, interferes with the synthesis of many secondary metabolites. In some microorganisms, the inhibitory effect of glucose has been related to a decrease in pH [3, 4, 10]. Haavik [6] reported that bacitracin production by *Bacillus subtilis* is pH dependent and that the inhibitory effect of glucose is due to acidification as a result of the accumulation of organic acids. In a previous study, we suggested that the effect of high concentrations of carbon sources upon prodigiosin production by *Serratia marcescens* could be an effect of the low pH produced by sugar catabolism [13]. We are reporting here that the decrease in pH of resting cells of *S. marcescens* after addition of a metabolizable sugar is due to the rapid production of lactic, acetic, citric and pyruvic acids. We have extended these observations by studying the acid production by bacteria which differ in their metabolisms of exogenous glucose. We used a method based on Stephan-curve pH change seen in dental plaque [14]. We measured the acidic pH response of resting-cell suspensions after a carbon source pulse. This method is rapid and simple and could be used in further studies on bacterial metabolism.

Material and methods

Bacterial strains and growth conditions The strains used were: *Serratia marcescens* Nima, ATCC 274, ATCC 43820 and N28b [5]; *Serratia liquefaciens* ATCC 27592; *Escherichia coli* ATCC 10536 and K12; *Pseudomonas aeruginosa* PAO 1 and ATCC 27853; and *Enterococcus faecalis* ATCC 10541 and ATCC 19433. *S. marcescens*, *S. liquefaciens*, *E. coli* and *P. aeruginosa* were grown in the complete medium described by Williams et al. [15]. Trypticase soy broth (TSB) was used for the growth of *E. faecalis*. Two colonies grown on trypticase soy agar (TSA) plates were inoculated into 500 ml Erlenmeyer flasks containing 100 ml of the selected media. The flasks were incubated without shaking at 37.5°C for 24 h. The cells were collected, washed three times in distilled water and suspended in 0.85% NaCl sterile solution to a suspension concentration of 0.7 to 0.8 mg of cell protein per mililiter.

To subject bacteria to a heat-shock, suspensions were heated for 10 min in boiling water, cooled, and used for the experiments.

Measurement of cell protein Protein content was determined according to Lowry et al. [9].

Sugar pulse studies Experiments were conducted on 8-ml samples of cell suspensions in 10-ml glass vials, which were

magnetically stirred at room temperature. After insertion of the pH electrode we mixed the suspensions vigorously with a small magnetic flea. The pH of such suspensions ranged from 5.7 to 6.0. After 2–4 min, a glucose pulse was given, usually as an 80 μL aliquot of concentrated solution in distilled water, and changes in external pH were recorded for 7 or 15 min. Glucose was added to the suspensions at concentrations described in the text and figures. Glycerol, fructose, dulcitol, galactose, lactose, maltose, sucrose and deoxyglucose pulses were added as 80 μL aliquots of 150 mM sterile solutions. Iodoacetate and carbonylcyanide m-chlorophenylhydrazone (CCCP) were added (5 mM final concentration), as small volumes of concentrated stocks in distilled water and acetone respectively, 15 min before the glucose pulse. All sugar-pulse studies were performed in duplicate, and results are expressed ± S.D. of three independent experiments.

Acid analysis A 2 ml aliquot of cell suspension was harvested and the supernatant was filtered twice through a 0.22 μm Millipore membrane and analysed directly by high pressure liquid chomatography (HPLC). The HPLC equipment was an Aminex HPX-87H (Bio-Rad cation exchange column) for organic acids operating at 35°C, using 0.01 M H_2SO_4 as eluent at a flow rate of 0.5 ml min^{-1}. The column effluents were monitored using an UV spectrophotometric detector at 210 nm. Peak height measurements with an external standard were used for quantification. Standards of the acids (analytical grade obtained from Fluka) were prepared individually in ultra pure water and chromatographed separately to determine the retention time for each acid. The acids were then chromatographed as a mixture. All determinations were carried out in duplicate, and the results are expressed ± SD.

Results and Discussion

Previously, we reported that the reduction in prodigiosin production by *Serratia marcescens* mediated by glucose and other metabolizable sugars was due to a decrease in pH observed in the cell suspensions [13]. Figure 1A shows that the addition of 1.5 mM glucose (final concentration) to cell suspensions of *S. marcescens* NIMA caused a rapid fall in pH (1.17 pH units in 7 minutes). We did not detect any change in pH when no sugar was added to the bacteria suspensions (control). When *S. marcescens* suspensions were subjected to a heat-shock, no cells could be grown, and the pH of these suspensions did not change after a sugar-pulse. The rate and magnitude of pH fall after a glucose-pulse depended on the concentration of the carbon source (Fig 1B): the more glucose was added the more pH decreased. With glucose concentrations from 0.015 mM to 0.15 mM the pH of bacteria suspensions decreased rapidly to a minimum, and then increased slowly. The lowest pH values were reached later in the experimental period, when the initial concentration of glucose was the highest. No significant differences were detected with glucose concentrations higher than 1.5 mM (data not shown).

In shaking cultures of *S. marcescens* containing high concentrations of glucose, most glucose is oxidized

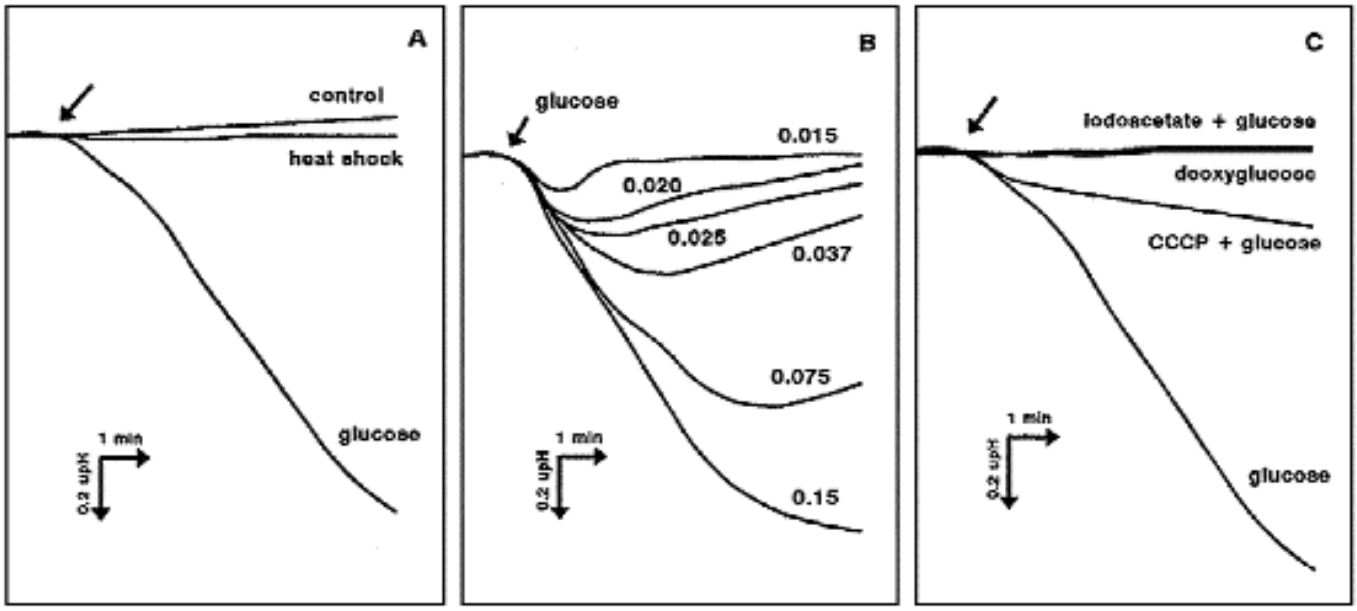

Fig. 1 (A) pH curves of NPC of *Serratia marcescens* with glucose 1.5 mM, without glucose (control) and glucose after a heat-shock. (B) Dependence of the rate of acidification on the concentration of added glucose (mM). (C) pH curves with deoxyglucose, glucose and glucose after addition of iodoacetate and CCCP. The addition of pulses was indicated by the arrow

extracellularly to gluconate, which accumulates in the medium, and pH falls below 3.5 [1]. This decrease in pH was not observed when deoxyglucose, a non-fermentable sugar analog of glucose which can be oxidized extracellularly to gluconic acid, was added (Fig. 1C). The same results were obtained when a glucose pulse was given to bacteria suspensions in the presence of iodoacetate, an inhibitor of glycolysis. These results indicate that pH changes in *S. marcescens* suspensions detected after glucose pulses were not due to extracellular oxidation of glucose. Moreover, the decrease in pH was not completely inhibited by treating cells with CCCP, a proton-conducting uncoupler. These results suggest that the pH was not due to proton extrusion through the respiratory chain after total oxidation of glucose [7]. The treatment with these chemical agents had no effect on the viability of bacteria. The number of colony-forming units (CFU) of cell suspensions at the end of each experiment was not significantly different from the number of CFU in control bacteria suspensions.

The pH changes for *S. marcescens* suspensions produced by different carbohydrates (1.5 mM, final concentration) are shown in Table 1. The decrease in external pH of these suspensions depended on the carbon source used. Glucose was the carbon source that induced the highest pH fall. Pulses of non-fermentable substrates, such as dulcitol and lactose, did not produce this pH fall.

Table 1 pH response of suspensions of *Serratia marcescens* pulsed with different carbohydrates (1.5 mM final concentration) after 7 and 15 minutes of the pulse

	^{3}pH 7 minutes	^{3}pH 15 minutes
Control	0.01 ± 0.00	0.06 ± 0.00
Glucose	–1.17 ± 0.01	–1.67 ± 0.01
Glycerol	–0.57 ± 0.00	–0.77 ± 0.01
Dulcitol	0.03 ± 0.00	0.05 ± 0.00
Fructose	–0.55 ± 0.02	–0.72 ± 0.01
Galactose	–0.01 ± 0.00	–0.09 ± 0.00
Lactose	–0.01± 0.00	0.03 ± 0.00
Maltose	–0.34 ± 0.01	–0.53 ± 0.00
Sucrose	–0.08 ± 0.01	–0.15 ± 0.02

Table 2 shows the magnitudes of pH decreases for bacteria which differ in their metabolism of glucose. The pH response of bacteria suspensions to a glucose-pulse depended on the type of bacteria. *Pseudomonas aeruginosa* is an obligately aerobic bacterium which oxidizes glucose extracellularly to gluconic and 2-ketogluconic acids. These acids are then translocated, phosphorylated and metabolized intracellularly via the Entner-Doudoroff pathway. *Escherichia coli* and *Serratia liquefaciens* are Enterobacteriaceae which oxidize glucose extracellularly only when the cofactor pyrrol-oquinoline quinone (PQQ) is added to suspensions. Their metabolisms can be either oxidative or fermentative but they differ in the type and proportion of fermentation products obtained by anaerobic fermentation of glucose. *E. coli* is a mixed acid fermenter and *S. liquefaciens* is a butanediol fermenter. *Enterococcus faecalis* is a homofermentative bacterium producing virtually a single fermentation product, lactic acid. As shown in Table 2, iodoacetate inhibited the pH fall after a glucose pulse for all the bacteria suspensions studied. The decrease in external pH was not observed when deoxyglucose was added to cell suspensions, except in the case of *E. coli*. Moreover, external pH of *E. faecalis* suspensions also decreased after a glucose pulse. These results confirmed our hypothesis that the pH fall of bacterial suspensions after a sugar pulse was not due to extracellular oxidation of glucose or proton extrusion.

Table 2 pH response of suspensions of different bacterial species pulsed with glucose, glucose and iodoacetate, and deoxyglucose 7 min after the pulse. The results are expressed in ^{3}pH

Microorganism	Glucose	Glucose + iodoacetate	Deoxyglucose
Pseudomonas aeruginosa	0.01 ± 0.00	0.04 ± 0.01	0.04 ± 0.01
Escherichia coli	–0.31 ± 0.00	–0.07 ± 0.00	–0.01 ± 0.00
Serratia liquefaciens	–0.82 ± 0.02	0.04 ± 0,02	0.02 ± 0.00
Enterococcus faecalis	–0.61 ± 0.01	0.04 ± 0.00	0.02 ± 0.00

We explored the possibility that the pH response was due to the production of organic acids. Seven and fifteen minutes after a glucose or glycerol pulse, we found citric, pyruvic, lactic and acetic acids in the supernatant of *S. marcescens* suspensions (Table 3). We did not detect these organic acids in the supernatants of bacterial suspensions treated with iodoacetate or subjected to deoxyglucose pulses. Nor did we find them in bacterial suspensions to which no sugar had been added. The relation between the pH fall and the relative amounts of organic acids detected in the supernatants was examined. pH Changes which took place when relative amounts of the organic acids presumed to have come from glucose and glycerol metabolism were added to *S. marcescens* suspensions were almost identical to those observed after pulses of these carbohydrates (data not shown).

Table 3 Concentrations (μM) of acids measured in suspension medium of *Serratia marcescens* after 7 and 15 min of glucose or glycerol pulses

Acids	Glucose		Glycerol	
	7 min	15 min	7 min	15 min
Citric	20 ± 2	32 ± 3	10 ± 1	17 ± 3
Pyruvic	50 ± 4	80 ± 6	4 ± 1	15 ± 2
Lactic	68± 2	125 ± 6	21 ± 2	42 ± 3
Acetic	66± 6	63 ± 1	80 ± 6	111 ± 7

The concentrations of citric, pyruvic, lactic and acetic acids found in the supernatants of suspensions of *S. marcescens*, *S. liquefaciens*, *E. faecalis*, *P. aeruginosa* and *E. coli* after a glucose-pulse are shown in Table 4. The rates at which these

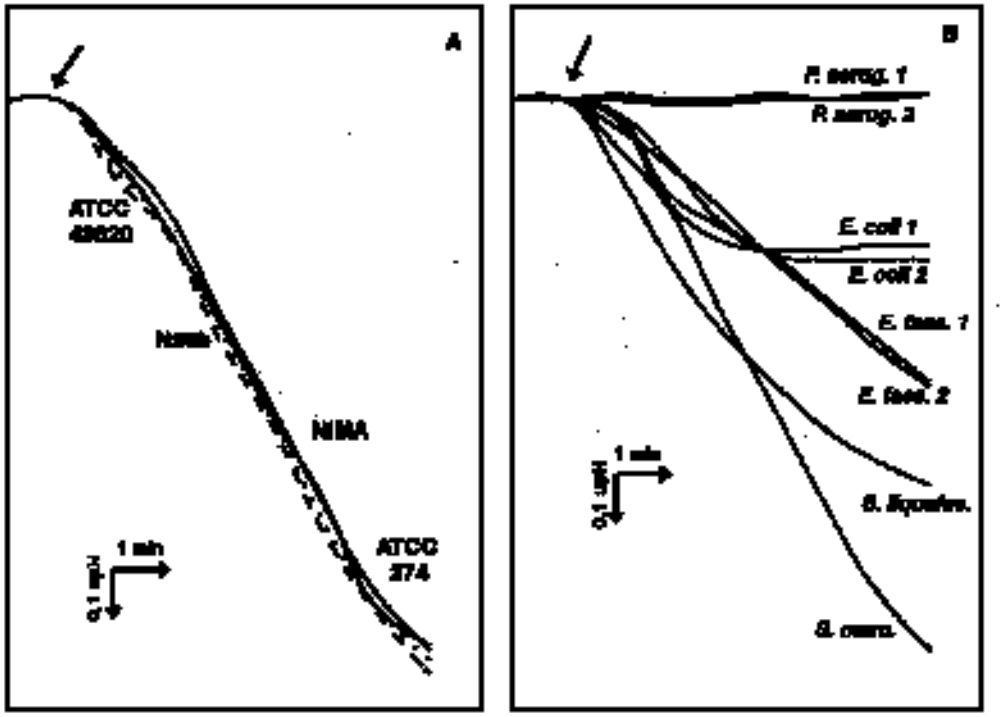

Fig. 2 (A) pH Response of suspensions of pigmented (Nima and ATCC 274) and non pigmented (ATCC 43820 and N28b) strains of *Serratia marcescens* after a glucose-pulse. (B) pH Response of different bacteria suspensions after a glucose-pulse. *P. aerug. 1= Pseudomonas aeruginosa* PAO1; *P. aerug. 2 = P. aeruginosa* ATCC27853; *E. coli 1 = Escherichia coli* ATCC10536; *E. coli 2 = E. coli* K12; *E. faec. 1 = E. faecalis* ATCC10541; *E. faec. 2 = E. faecalis* ATCC19433; *S. liquefac. = Serratia liquefaciens* ATCC27592; *S. marc. = Serratia marcescens* NIMA

organic acids originated determined the pH of these bacterial suspensions after seven minutes of incubation (see also Fig 2B).

To determine whether the pH fall of *S. marcescens* suspensions after a glucose-pulse depended on the ability of the strain to produce prodigiosin, non-proliferating suspensions of pigmented (NIMA and ATCC 274) and non pigmented (ATCC 43820 and N28b) strains of *S. marcescens* were submitted to the same experimental conditions. As shown in Fig. 2A, all *S. marcescens* strains showed similar pH curves after a glucose pulse. Fig. 2B shows the pH fall for suspensions of strains of *S. marcescens*, *S. liquefaciens*, *E. faecalis*, *E. coli* and *P. aeruginosa* after a sugar pulse. No significant differences between pH curves for strains of the same bacteria species were observed after a glucose pulse. The pH response to a sugar stimulus was different for each bacterial species studied. These results confirmed the reproducible nature of the fall in pH of bacterial suspensions induced by sugar solution.

These results suggest that the rapid acidification of the medium after a sugar-pulse could be due to the presence of organic acids resulting from bacterial metabolism. It has been shown that glucose and other metabolizable sugars interfere with the synthesis of many secondary metabolites [2–4, 6, 8, 10, 11, 13]. This study has established a methodology which will enable to determine which carbohydrates can be used as carbon sources in the production of secondary metabolites, by studying the pH response of bacterial suspensions to sugar stimuli. Carbohydrates which produce rapid acidification of the medium may not be good carbon sources in studies on secondary metabolism. This study has demonstrated the feasibility of determining, with relative simplicity, a repeatable response of bacteria suspensions to sugar stimulus.

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Table 4 Concentration (μM) of acids measured in suspension medium of different bacterial species after 7 min of a glucose pulse

Acids	*S. marcescens**	*S. liquefaciens*	*E. faecalis*	*E. coli*	*P. aeruginosa*
Citric	20 ± 2	10 ± 2	ND	ND	ND
Pyruvic	50 ± 4	4 ± 1	1 ± 1	ND	ND
Lactic	68 ± 2	16± 1	21 ± 2	8 ± 2	ND
Acetic	66 ± 6	36± 6	6 ± 2	5 ± 2	ND

ND = Not detected.
* Genera as in Table 1.

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