Sodium-dependent Growth and Respiration of a Nonhalophilic Bacterium, *Pseudomonas stutzeri*

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SUMMARY

Pseudomonas stutzeri (van Niel strain) requires Na^+ for growth. Its growth rate was a sigmoidal function of Na^+ concentration, being maximal and constant from 2 to 50 mM- Na^+ , and half maximal at about 0.5 mM- Na^+ . The relationship between cell concentration and Na^+ concentration was non-linear; cell concentration increased abruptly when Na^+ was greater than 0.3 mM. Accumulation of Na^+ in the organism during growth was not detected. In the presence of K^+ , respiration was enhanced specifically by Na^+ . The respiration rate of the organism growing in the culture was a linear function of the growth rate when limited by the Na^+ concentration, whereas the maximum rate induced by excess Na^+ was independent of the growth rate.

INTRODUCTION

Micro-organisms showing optimal growth in aqueous environments containing less than 2 % (w/v) NaCl are termed nonhalophiles (Larsen, 1962). Usually they do not need Na^+ for growth, but recently there have been several reports that certain enteric bacteria require Na^+ under particular growth conditions. In some of these bacteria, the requirement for the cation can be correlated with a single metabolic step catalysed by a specific Na^+ -activated enzyme (O'Brien & Stern, 1969*a, b, c*). In others, Na^+ together with K^+ has been claimed to be involved in the transport of specific substrates (Frank & Hopkins, 1969; Halpern *et al.*, 1973; Stock & Roseman, 1971). As is described in this paper, however, the requirement of *Pseudomonas stutzeri* for Na^+ is absolute. The cation is indispensable for growth irrespective of the carbon and nitrogen source and the nature of the terminal electron acceptor. In addition, the requirement is met at a relatively low level of Na^+ which has little osmotic effect.

We have attempted to characterize the Na^+ requirement of this organism by comparing it with the K^+ requirement. We have also investigated the effects of Na^+ on respiration. The results reveal a unique physiological response of *P. stutzeri* to Na^+ and suggest a possible involvement of the cation in the regulation of energy metabolism.

METHODS

Organism. A van Niel strain of *Pseudomonas stutzeri* (IAM12097) was maintained on nutrient agar slopes containing 20 mM- KNO_3 , and transferred at monthly intervals as described previously (Kodama, Shimada & Mori, 1969).

Culture media. In all experiments, except those for studying growth on other carbon or nitrogen sources, TLG medium of the following composition was used: 100 mM-Tris;

10 mM-phosphoric acid; 40 mM DL-lactic acid; 30 mM L-glutamic acid; 2 % (v/v) trace element mixture (Sistrom, 1960); the pH was adjusted to 7.2 with concentrated HCl. This medium was designed to support and control the rapid growth provided that an appropriate amount of Na^+ and K^+ was added. Phosphate medium contained: 40 mM-potassium phosphate buffer pH 7.2; 15 mM- $(\text{NH}_4)_2\text{SO}_4$; 2 % (v/v) trace element mixture. This medium was supplemented with appropriate carbon and/or nitrogen sources. Endogenous concentrations of Na^+ and K^+ in TLG medium prepared routinely were usually less than 30 μM and 5 μM , respectively. The Na^+ concentration in phosphate medium was comparable to that in TLG medium. Na^+ and K^+ concentrations were determined in a Hitachi 204 atomic absorption spectrometer. The potassium salts of organic acids were prepared by titration to pH 7.2 with KOH. For preparation of a nutrient broth with a minimal Na^+ content, concentrated nutrient-broth solution containing 10 g trypticase (BBL), 5 g beef extract (Oxoid) and 1 g yeast extract (Daigo Nutritive Chemicals, Osaka, Japan) per 100 ml water was passed slowly through a column (1 $\times$ 15 cm) containing Dowex 50 $\times$ 2 (H^+ -form). When elution of the coloured solution began, the Na^+ concentration in each fraction (1 ml) of the eluate was measured, and fractions with less than 0.5 mM- Na^+ were combined, neutralized with KOH and stored after autoclaving. The stock solution was diluted fivefold with the phosphate medium before use.

Culture conditions. Culture vessels were either glass test-tubes (18 $\times$ 200 mm) fitted with Morton closures (Bellco Glass, Vineland, New Jersey, U.S.A.) or 100 ml Erlenmeyer flasks capped with aluminium foil; all had a photometric side-tube for absorbance measurements of the culture. The test-tubes and flasks contained 5 ml and 20 ml of the medium, respectively. All cultures were incubated at 30 °C in a Taiyo M-1N reciprocating shaker (Taiyo Scientific Co., Tokyo, Japan) operated at 120 strokes/min and with a 5 cm stroke length.

Bacterial mass determination. This was estimated from the absorbance of cultures or washed bacterial suspensions in a Klett-Summerson colorimeter with a no. 66 red filter. The relationship between concentration and absorbance was linear up to 100 Klett units (one Klett unit = 3.7 μg dry wt organisms ml^{-1}). This relationship did not change significantly for organisms grown at different rates. The dry weight was determined according to the method of Herbert, Phipps & Strange (1971).

Measurement of growth rate. The cultures were grown, monitored by measuring their absorbance and repeatedly diluted with fresh medium. A constant rate of growth was established after two or three dilutions: this state is referred to as a balanced growth. Once balanced growth was established, the growth rate was constant as long as the absorbance of the culture was kept below 60 Klett units by dilution with fresh medium. The growth rate is expressed as specific growth rate $\mu = 1/\tau \ln 2 = 0.69/\tau$, where τ is the doubling time (h).

Na^+ and K^+ requirement tests. The media used to assess the requirement of organisms for Na^+ were prepared by adding 10 mM-KCl to TLG medium or 30 mM carbon or nitrogen sources to phosphate medium (except in the case of the Dowex-treated nutrient broth). Cultures grown overnight in the same medium as that to be used for the test, and washed at least twice with the medium which was free of added Na^+ , were used as the inoculum. The K^+ requirement was assessed using TLG medium supplemented with 10 mM-NaCl. The effects of Li, Rb, Cs and choline were tested by adding each one as its chloride.

Measurement of respiration. The culture in a state of balanced growth was quickly chilled in an ice-bath. Organisms were harvested by centrifuging at 5000 g for 3 min (4 °C), washed twice with cold TLG medium which was free of added K^+ and Na^+ , and resuspended in the same medium and stored at 0 °C. When this suspension was used as the inoculum within 6 h, exponential growth commenced without any appreciable lag period. Respiration was

measured at 30 °C with an oxygen electrode respirometer (Yellow Springs Instruments Yellow Springs, Ohio, U.S.A.).

The respiration rate of organisms growing under Na^+ -limited conditions was measured by transferring samples from the culture vessels into the respirometer and following the oxygen uptake, starting within 15 to 20 s after sampling. As oxygen was taken up by a zero order reaction until the oxygen in the culture medium was almost exhausted and the rate constant was proportional to the bacterial concentration in the culture, the procedure is satisfactory for measuring the respiration rate of organisms growing in the culture vessel (referred to as '*in situ*' Q_{O_2} in this paper). The potential respiration rate was measured by following the oxygen uptake, as described above, after the addition of excess Na^+ (5 to 10 mM) to the sample.

Chemicals. All reagents were of the highest grade commercially available. KOH (Na^+ content less than 0.002 % (w/w)) and 'Suprapur' grade NaCl were purchased from Merck (Darmstadt, West Germany).

RESULTS

Na⁺ requirement for growth

The dependence of aerobic growth of *P. stutzeri* on alkaline metals was tested using TLG medium. As reported for many other bacteria (Tempest, 1969), the K^+ requirement was met by Rb^+ in this bacterium. However, neither Cs^+ nor Li^+ could replace K^+ . Even in the presence of K^+ , there was no appreciable growth in the absence of Na^+ . Adding 5 mM-NaCl to the deficient culture immediately elicited growth at a rate comparable with that of the culture supplemented with NaCl at inoculation. LiCl, RbCl or CsCl at 10 mM failed to support growth when added to the Na^+ -free medium, but they did not inhibit growth supported by 10 mM-NaCl. NH_4^+ and choline ion were also unable to support growth in the absence of Na^+ .

With cultures in phosphate media with different carbon and/or nitrogen sources (glycerol, glucose, maltose, lactose, acetate, malate, glutamate and nutrient broth), no growth was observed 24 h and 48 h after inoculation unless NaCl was added. Na^+ was also required for anaerobic growth of *P. stutzeri* in the presence of nitrate. Thus, among the alkaline metals, Na^+ , as well as K^+ , was indispensable for the growth of this organism regardless of the nature of the carbon and nitrogen sources and the terminal electron acceptors. This organism is therefore similar to *Rhodopseudomonas spheroides*, which requires Na^+ for both light-anaerobic and dark-aerobic growth, irrespective of the nature of the carbon or nitrogen sources (Sistrom, 1960; Taniguchi & Kikuchi, unpublished results).

Effects of Na⁺ concentration on growth

Pseudomonas stutzeri grew at a constant rate in medium containing from 2 to 50 mM-NaCl in the presence of 50 mM-KCl. (Fig. 1). The growth rate markedly decreased with NaCl above 200 mM. With higher levels of KCl (120 mM), the decrease in growth rate was noticeable even at 30 mM-NaCl. These observations confirm that *P. stutzeri* is a nonhalophile.

With low Na^+ concentrations in TLG medium supplemented with 10 mM-KCl the growth rate was a sigmoidal function of Na^+ concentration (Fig. 2*a*), whereas with varying concentrations of K^+ , and Na^+ held at 10 mM, the growth rate was a hyperbolic function of the K^+ concentration (Fig. 3*a*) and thus gave the type of relationship which is generally observed when either the carbon or the nitrogen source limits growth (Monod, 1942). (The changes in the osmolarity and ionic strength of the medium caused by adding NaCl and

Fig. 1. Effect of NaCl concentration on the growth of *Pseudomonas stutzeri* in TLG medium plus KCl at: ○, 10 mM; ●, 50 mM; △, 80 mM; ▲, 120 mM.

Fig. 2

Fig. 2. Effect of Na⁺ concentration on the growth of *Pseudomonas stutzeri* in TLG medium plus 10 mM-KCl: (a) specific growth rate; (b) cell mass.

Fig. 3

Fig. 3. Effect of K⁺ concentration on the growth of *Pseudomonas stutzeri* in TLG medium plus 10 mM-NaCl: (a) specific growth rate; (b) cell mass.

KCl were negligible when compared with the total ionic concentration of approximately 200 mM for TLG medium.)

From the linear relationship between K⁺ concentration and cell mass (Fig. 3b), the molar growth yield for K⁺ (Y_{K^+}) can be calculated to be 2.8×10^3 g organisms. On the other hand the cell mass exhibits a distinctive non-linear response to the increased Na⁺ concentrations

Fig. 4

Fig. 5

Fig. 4. Changes in K^+ concentration during the growth of *Pseudomonas stutzeri*. Samples of a growing culture were taken at intervals, filtered through a membrane filter MF 50 (pore size $0.45 \mu m$; Sartorius, Göttingen, West Germany), and the concentrations of K^+ in the filtrates were measured by atomic absorption spectrometry. Initial K^+ concentrations were: ●, $490 \mu M$; ○, $82 \mu M$.

Fig. 5. Effect of K^+ and Na^+ on the respiration of *Pseudomonas stutzeri* in TLG medium. Reactions were started by the addition of washed cell suspensions to TLG medium without added K^+ and Na^+ . Other additions were made as indicated.

(Fig. 2b). A small increment of Na^+ concentration around $0.3 \text{ mM-}Na^+$ provokes some 50-fold increase in the cell mass.

Na^+ and K^+ were also different in the rates at which they disappeared from the medium during growth: the K^+ concentration decreased linearly (Fig. 4), whereas the Na^+ concentration remained unchanged within the limits of experimental accuracy. The decrease in K^+ concentration per unit increase in bacterial mass was 0.33 and $0.36 \mu M\text{-}K^+$ per μg organisms per ml for initial K^+ concentrations of 490 and $82 \mu M$, respectively. From these values, which agree with the reciprocal of the Y_{K^+} value, the K^+ concentration in the organisms can be estimated to be approximately 150 mM , assuming the intracellular water content to be 2.5 g per g bacterial mass (Schultz & Solomon, 1961). Estimation of the intracellular Na^+ content by the same method is impossible.

Effects of Na^+ on respiration

The effects of Na^+ on respiration were studied to establish a reason for its requirement for the growth of *P. stutzeri*. To compare directly the Na^+ effect on respiration with that on growth, respiration was measured in the culture medium using cells potentially capable of growing (Fig. 5). Clearly, respiration proceeded at an appreciable rate in the absence of added K^+ and Na^+ . However, the oxygen uptake rate was enhanced about 1.5 times by K^+ , and doubled when Na^+ was added subsequently. No enhancement resulted when Na^+ was added without simultaneous addition of K^+ . Thus, K^+ is necessary for the enhancing function of Na^+ . Other alkaline metals, added as chlorides in place of $NaCl$, did not stimulate respiration.

Stimulation of respiration by Na^+ was concentration dependent within the range that limits growth (Fig. 6). The maximum stimulation was attained at cation concentrations above 2 mM and the half maximum at about 0.5 mM .

Fig. 6

Fig. 7

Fig. 6. Effect of Na^+ concentration on the respiration of *Pseudomonas stutzeri* in TLG medium plus 10 mM-KCl. The growth rate was limited by the Na^+ concentration in the medium. Specific growth rates (h^{-1}) were: ●, 0.69; ■, 0.57; ○, 0.46.

Fig. 7. The correlation between growth rate and Q_{O_2} of *Pseudomonas stutzeri* under Na^+ -limited conditions: ○, potential Q_{O_2} ; ●, *in situ* Q_{O_2} . Both lines were drawn by regression analysis.

Respiratory activity under Na^+ -limited conditions

The above results led us to study the correlation between respiration rate and growth rate under Na^+ -limited conditions (Fig. 7). The potential Q_{O_2} (i.e. the maximum respiratory activity of growing cells brought about by excess Na^+) was independent of the growth rate, which implies that the Na^+ concentration does not affect the potential level of the respiratory system. On the other hand, the *in situ* Q_{O_2} increased linearly with the growth rate, which would be expected if the growth rate was energetically limited according to the equation

$$Q_{\text{O}_2} = \mu/Y_{\text{O}_2} + m_{\text{O}_2}$$

in which Q_{O_2} is the specific respiration rate ($\text{mol g}^{-1} \text{h}^{-1}$), Y_{O_2} is the cell yield coefficient for oxygen (g mol^{-1}), and m_{O_2} is the apparent maintenance respiration rate ($\text{mol g}^{-1} \text{h}^{-1}$), equal to the respiration rate at $\mu = 0$. Thus the data suggest that Na^+ is involved in the control of respiration in growing organisms.

DISCUSSION

There are several hypotheses which could explain the Na^+ requirement of *Pseudomonas stutzeri*.

1. A single step in intermediary metabolism, which is catalysed by a specific Na^+ -activated enzyme, could be rate-limiting for growth. This hypothesis cannot yet be ruled out, but it does not appear to provide an explanation for the rapid response of respiration to added Na^+ in the presence of K^+ .

2. A more attractive hypothesis is one based on Na^+ plus K^+ -activated transport, as found in a halophilic pseudomonad which shows an obligate Na^+ -requirement (MacLeod, 1965; Thompson & MacLeod, 1971). However, the Na^+ -activated transport hypothesis alone can hardly account for the failure of *P. stutzeri* to grow under K^+ -rich but Na^+ -deficient

conditions, because even under such conditions the respiration rate is as high as one third to one half of the maximum rate brought about by excess Na^+ . This suggests a qualitative difference between the respiration of organisms under growing conditions (with both Na^+ and K^+) and the respiration of those prevented from growing by lack of Na^+ in spite of the presence of other nutrients.

3. Another hypothesis is that energy metabolism is coupled to respiration only in the presence of both Na^+ and K^+ . High respiration rates with both cations could be explained as a result of the energetic coupling and energy-dependent transport of substrates. According to this hypothesis, the linear relationship between the *in situ* Q_{O_2} and the growth rate under Na^+ -limited conditions (Fig. 7) could also be interpreted as indicating that the activity of the respiratory system controlled by the cation concentration is rate-limiting for growth and/or the respiratory activity is controlled through energy demand limited by Na^+ concentration. Neither explanation can be ruled out at present.

Hypothesis 3 seems most probable because we have found that all energy-dependent functions hitherto tested, such as K^+ , amino-acid and phosphate transport and cell motility, are apparently Na^+ -dependent. Furthermore, uncouplers of oxidative phosphorylation, such as 2,4-dinitrophenol and carbonylcyanide *m*-chlorophenylhydrazone, stimulate respiration in the absence of either Na^+ or K^+ , suggesting that respiration is in a restrained state when not coupled to energy-dependent functions. Details of these results will be reported elsewhere. The mechanism by which Na^+ and K^+ facilitate the energetic coupling remains to be elucidated.

Finally, it should be emphasized that the growth rate of *P. stutzeri* can be controlled not only by supplying different carbon or nitrogen sources, but also simply by limiting the Na^+ concentration in a permissive medium having a chemically defined composition. This means that it is possible to make approaches to several problems related to bacterial growth using this system. A change of Na^+ concentration will, without disturbing the major nutritional and ionic conditions, produce unique shifts, for example, dilution decreases the growth rate (shift-down) and addition of NaCl increases the growth rate (shift-up).

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