The Basis of the Alkalophilic Property of a Species of *Bacillus*

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SUMMARY

An alkalophilic bacterium belonging to the genus *Bacillus* was isolated from an indigo ball. The bacterium exhibited a maximum growth rate at pH 10.0 to 10.5. The incorporation of ^{14}C -labelled amino acids or ^{14}C uracil, uptake of ^{14}C -labelled α -amino isobutyric acid into the bacterium and oxygen consumption of the bacterium with amino acids as substrates were all maximum at pH 9.0 to 10.5. The uptake of $[\text{U-}^{14}\text{C}]$ glucose into the organism and oxygen consumption with carbohydrates, on the other hand, showed little variation of rate in the pH 8 to 10 region. The oxygen consumption of intact bacteria or protoplasts in culture medium was maximum at pH 10. The membrane of the bacterium oxidized NADH maximally at pH 7.5, and ATPase bound to the membrane exhibited maximum activity at pH 7. L-Lactate, L-alanine and malate dehydrogenases in the soluble fraction exhibited maximum activities at pH 7.4 to 8.4. The alkalophilic property of the bacterium may be due to the behaviour of the membrane towards charged substances admitted into the organisms.

INTRODUCTION

Recently, many species of alkalophilic bacteria belonging to the genus *Bacillus* have been isolated (Horikoshi, 1971 *a, b*, 1972; Boyer & Ingle, 1972; Horikoshi & Atsukawa, 1973 *a, b*; Kurono & Horikoshi, 1973). These bacteria were found to prefer alkaline environments (pH 10 to 11) for their optimum growth. The authors cited above have purified various kinds of exoenzymes from the alkalophilic bacteria and examined the biochemical properties, especially those concerning the alkalophilicity and alkalostability, of the enzymes. No work on the alkalophilic properties or alkalostability of the bacteria, however, has yet been reported. In our laboratory an alkalophilic bacterium exhibiting a maximum growth rate at pH 10.0 to 10.5 was isolated from an indigo ball, which mainly consists of fermented indigo leaves and is used as a source of natural indigo dye. We report some biochemical properties concerning the alkalophilicity and alkalostability of the bacterium.

METHODS

Media. For isolation and maintenance of the organism, a medium of the following composition was used (g/l deionized water): polypeptone, 10; meat extract, 10; yeast extract, 3; glucose, 3; Na_2CO_3 (anhydrous), 10. Solid medium was prepared by the addition of 2% (w/v) agar. For culture, a medium of the following composition was used (g/l deionized water): polypeptone, 10; glucose, 10; yeast extract, 1.5; $\text{Na}_2\text{PO}_4 \cdot 12\text{H}_2\text{O}$, 1.5; NaCl, 1.5; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1; 3 M-NaOH, 12.5 ml. The Na_2CO_3 or NaOH and the solution containing the other components were separately sterilized and mixed before incubation. The pH values of the media were 10.0 to 10.5.

Isolation. The indigo ball used as the source of the isolate was kindly given by Mr H. Ichimura, Ibaragi Prefecture, Japan. The material was inoculated into the isolation medium

and incubated at 37 °C with shaking, the pH value being kept at 10 to 11 with 3 M-NaOH during the incubation. After 2 days, the culture was streaked on the solid medium. After repeated transfer and plating-out on the solid medium at 37 °C, two different kinds of culture were obtained. One was able to grow well even at pH 7 to 8, while the other grew poorly. The latter culture was used in the present study.

Identification. The isolate was identified according to the methods described by Smith, Gordon & Clark (1952) and in *Bergey's Manual of Determinative Bacteriology* (1957).

Culture. The organisms from a fresh culture on solid medium (37 °C, 16 h) were inoculated in the culture medium (500 ml), and incubated at 37 °C with shaking, the pH being kept at 10.0 to 10.5 with 3 M-NaOH. The organisms in an early stationary phase (5 to 9 h) were collected by centrifuging, washed with 0.1 M-phosphate buffer (pH 7.5) containing 0.9 % (w/v) NaCl, and suspended in the buffer.

Protoplast, extract and the membrane and soluble fraction of the isolate. The organisms in early stationary phase were suspended in 20 mM-phosphate buffer (pH 7.3) containing 10 mM-MgCl₂ and 0.3 M-sucrose ($E_{650} = 50$; a suspension of $E_{650} = 10$ contained 5.5 mg dry wt organisms/ml). The suspension was treated with lysozyme (EC. 3.2.1.17; 50 µg/ml) at 30 °C for 10 min, and centrifuged at 5000 g for 10 min. The precipitate (protoplast) was washed with the buffer and suspended in the buffer (half the volume used to prepare the original suspension). The new suspension was used as the protoplast sample.

To the precipitate (protoplast) obtained as described above was added 20 mM-phosphate buffer (pH 7.3) containing 0.9 % (w/v) NaCl (about 15 times the volume of the precipitate) and then deoxyribonuclease I (EC. 3.1.4.5; 30 µg/ml). The mixture was incubated at 30 °C for 30 min and centrifuged at 30000 g for 40 min. The precipitate and supernatant liquid thus obtained were used as the membrane and soluble fractions, respectively. The precipitate was suspended in 20 mM-phosphate buffer (pH 7.3) containing 0.9 % (w/v) NaCl.

Base composition of deoxyribonucleic acid. DNA was isolated according to the procedure of Marmur (1961), and the guanine-cytosine (GC) content of the DNA was determined by the thermal denaturation method (Marmur & Doty, 1962).

Oxygen consumption. The oxygen consumption of intact bacteria with or without glucose (or L-amino acids) was manometrically measured in a Warburg flask. To 2.0 ml of the reaction mixture consisting of 10 mM-phosphate buffer, 0.9 % (w/v) NaCl and 0.3 % (w/v) Casamino acids (or 0.1 M-carbohydrates or 30 mM-L-amino acids) was added 0.2 ml of the suspended bacteria ($E_{650} = 13$). The pH value of the reaction mixture was previously adjusted with 3 M-NaOH to give a desired value when mixed with the suspension.

The oxygen consumption of protoplasts in the culture medium, or of extract with carbohydrates or amino acids as substrates, or of the membrane fraction with NADH as substrate, was measured at 37 °C using a Clark-type oxygen electrode. To 6.0 ml of the culture medium containing 0.2 M-sucrose was added 0.5 ml of the protoplast sample. To 6.0 ml of the reaction mixture, consisting of 10 mM-phosphate buffer, 0.9 % (w/v) NaCl and 10 mM-carbohydrates, amino acids or NADH, was added 0.5 ml of the extract or membrane fraction. The final pH of the mixture was as indicated in the text.

Enzyme assays. L-Lactate, L-alanine and malate dehydrogenases (EC. 1.1.1.27, EC. 1.4.1.1 and EC. 1.1.1.37, respectively) were assayed according to the methods of Hakala, Glaid & Schwert (1956), Wiame, Piérard & Ramos (1962) and Ochoa (1955), respectively. The procedure of Nakao, Tashima, Nagano & Nakao (1965) was used to assay ATPase (EC. 3.6.1.3).

Incorporation of amino acids into protein. The reaction mixture contained 0.1 M-glucose, 0.04 % (w/v) Casamino acids, 1 µCi of U-¹⁴C-labelled *Chlorella* hydrolysate (40.3 mCi/mg-

atom C) and 0.9 % (w/v) NaCl in 0.1 M-phosphate buffer of an appropriate pH value; 2.0 ml was pre-incubated at 37 °C for 3 min. The reaction was started by adding 0.5 ml of the bacterial suspension ($E_{650} = 10$), and terminated by adding 5 ml of cold 10 % (w/v) trichloroacetic acid (TCA) containing 0.032 % (w/v) Casamino acids. The precipitates were dissolved in 0.5 ml of 1 M-NaOH containing 1 % (w/v) thioglycolic acid. To the solution was added 5 ml of 5 % (w/v) TCA, and it was heated at 90 °C for 30 min. The precipitates were washed once with 5 % (w/v) TCA, twice with 5 ml acetone and twice with 5 ml ethanol, and were dissolved in a mixture of 0.2 ml of 1 M-NaOH and 15 ml of the scintillation fluid [4 g 2,5-diphenyloxazole (PPO), 0.1 g 1,4-bis(2-(5-phenyloxazolyl))-benzene (POPOP) in 1 l of a mixture of 70 % (v/v) toluene and 30 % (v/v) ethanol.] Radioactivity was measured in a Packard-Tris-Carb liquid scintillation spectrometer, model 3320.

Incorporation of uracil into nucleic acid. The procedure was essentially similar to that used for the incorporation of amino acids into protein, except that 1 μ Ci of [2- 14 C]uracil (21.0 mCi/mmol) was used in place of 14 C-labelled *Chlorella* hydrolysate and Casamino acids. The reaction was terminated with 10 % (w/v) TCA. The precipitates were washed with TCA and organic solvents and dissolved in a mixture of NaOH and the scintillation fluid, as described in the amino acids incorporation experiments.

Uptake of α -amino-isobutyric acid or glucose into bacteria. The reaction mixture used for the uptake experiments of α -aminoisobutyric acid (AIB) or glucose contained 0.1 M-glucose, 10 μ M-AIB and 2 μ Ci of 1- 14 C-labelled AIB (4.84 mCi/mmol) or 0.2 mM-glucose and 0.4 μ Ci of [U- 14 C]glucose (5.0 mCi/mmol), respectively, in 0.1 M-phosphate buffer containing 0.9 % (w/v) NaCl. To 1.5 ml of the reaction mixture pre-incubated for 3 min at 37 °C was added 0.5 ml of the bacterial suspension ($E_{650} = 10$). The pH value of the reaction mixture was previously adjusted with 3 M-NaOH to become a desired value when mixed with the suspension. After an appropriate reaction period at 37 °C, 1 ml of the suspension was removed and filtered through a Millipore filter (grade HA). The organisms on the filter membrane were washed three times with 2 ml of the reaction mixture from which 1- 14 C-labelled AIB or [U- 14 C]glucose was omitted. The reaction mixture used for washing had the same pH value as that at which the reaction was carried out. The cells with the membrane were dried overnight in a desiccator, and the radioactivity of the bacteria suspended in 15 ml of the scintillation fluid (4 g PPO and 0.1 g POPOP in 1 l of a mixture of 45 % v/v, methyl cellosolve and 55 % v/v, toluene) was measured.

Chemicals. Polypeptone and meat extract were purchased from Wako Pure Chemical Industries (Tokyo); yeast extract from Kyokuto Seiyaku Co. (Tokyo); vitamin-free Casamino acids from Nissui Seiyaku Co. (Tokyo); and L-amino acids from Ajinomoto Co. (Tokyo). Deoxyribonuclease and NADH were purchased from Sigma and lysozyme from Seikagaku Co. (Tokyo). Radioisotope-labelled compounds were all purchased from Daiichi Pure Chemicals Co. (Tokyo). Other chemicals were reagent grade.

RESULTS

Morphological and physiological characteristics of the isolate

The isolate was an aerobic, sporeforming (oval, central spores), Gram-positive, motile and rod-shaped bacterium (0.5 to 0.6 μ m $\times$ 2 to 3.5 μ m). The GC content of the DNA of the isolate was 42.8 %. The isolate therefore belongs to the genus *Bacillus* (*Bergey's Manual of Determinative Bacteriology* 1957; Smith, Gordon & Clark, 1952; Normore, 1973). The characteristic feature of the isolate was that the organism grew well in alkaline media and that the growth rate was best at pH 10.0 to 10.5. The growth rate was slight or zero at

Fig. 1

Fig. 2

Fig. 1. Incorporation of (○) ^{14}C -labelled amino acids or (●) ^{14}C uracil into an alkalophilic bacterium at different pH values.

Fig. 2. Oxygen consumption of intact bacteria or protoplasts of an alkalophilic bacterium at different pH values. ○, Intact bacteria with a culture medium; ●, protoplasts with a culture medium containing 0.2 M-sucrose. The activities were expressed in $\mu\text{l O}_2/\text{h/mg dry wt}$ (○), or $\mu\text{l O}_2/\text{h/mg protein}$ (●).

pH 7 to 8. The temperature range at pH 10.5 was 15 to 45 °C, and optimum temperature was 35 to 40 °C. The bacterium (pH 10 to 10.5; 37 °C) produced nitrites from nitrates, hydrolysed starch and gelatin, and was catalase positive. The organism did not produce H_2S and was Voges-Proskauer negative.

The pH dependencies of physiological activities of the isolate

The growth rate of the isolate was maximum at pH 10.0 to 10.5. Some physiological activities, such as synthesis of protein and nucleic acid, and oxygen consumption in the culture medium of intact bacteria may therefore be expected to be maximum also in this pH region.

The rate of protein or nucleic acid synthesis was estimated by the incorporation of ^{14}C -labelled amino acids or ^{14}C uracil into the TCA-insoluble fraction. The incorporation of amino acid into protein proceeded at a linear rate for about 20 min of incubation at either pH 7.0 or 10.0. The radioactivities of the bacteria incubated for 5 min were then measured at different pH values. The rate of incorporation of uracil into nucleic acid was linear for at least 3 min of incubation at either pH 7.0 or 10.0. The experiments at different pH values were then carried out with the organisms incubated for 3 min. The activity of protein or nucleic acid synthesis of the bacterium was maximum at about pH 10, or pH 9.0 to 9.5, respectively (Fig. 1).

The bacteria suspended in 0.1 M-phosphate buffer containing 0.9 % (w/v) NaCl consumed oxygen appreciably (20 to 30 $\mu\text{l O}_2/\text{h/mg dry wt}$ organisms), and the rate of oxygen consumption was almost the same in the pH 7 to 10.5 region. When the bacteria were suspended in the culture medium, increased oxygen consumption occurred and the activity was maximum at pH 10.0 to 10.5 (Fig. 2). The protoplast sample in 20 mM-phosphate buffer containing 10 mM- MgCl_2 and 0.2 M-sucrose exhibited no oxygen consumption in the pH 7 to

Fig. 3

Fig. 4

Fig. 3. Activities of (○) ATPase of the membrane, and (●) L-alanine dehydrogenase in the soluble fraction of an alkalophilic bacterium at different pH values.

Fig. 4. Oxygen consumption of an alkalophilic bacterium with amino acids at different pH values. ○, Casamino acids; ●, alanine; △, serine; ▲, glutamic acid.

10.5 region. When suspended in the culture medium containing 0.2 M-sucrose, the proto-plasts consumed oxygen rapidly, and the activity was also maximum at pH 10.

When NADH was added to the membrane fraction, oxygen was consumed rapidly. The activity was maximum at pH 7.5 ($120 \mu\text{l O}_2/\text{h/mg protein}$) and no activity was observed at pH 10. The ATPase bound to the membrane also exhibited maximum activity at pH 7, and no activity was observed at pH 10 (Fig. 3). L-Alanine dehydrogenase in the soluble fraction of the bacteria exhibited maximum activity at pH 7.8 and no activity at pH 10 (Fig. 3). L-Lactate and malate dehydrogenases in the soluble fraction exhibited maximum activities at pH 7.4 and 8 (50 and $0.15 \mu\text{mol NADH}$, respectively, oxidized/min/mg protein) and less activity at pH 10 (15 and $0.04 \mu\text{mol NADH}$, respectively, oxidized/min/mg protein). These results suggest that the intracellular pH of the bacteria may be about neutral and that the alkalophilic properties of the biochemical reactions of intact bacteria may depend on the membrane function.

The pH dependencies of oxygen consumption of the isolate with various substances

Although the bacteria suspended in 0.1 M-phosphate buffer (pH 10.0) containing 0.9 % (w/v) NaCl consumed oxygen (20 to $30 \mu\text{l O}_2/\text{h/mg dry wt}$), addition of glucose (0.1 M) increased this by 90 to $100 \mu\text{l O}_2/\text{h/mg dry wt}$ organisms. Addition of other carbohydrates such as fructose and glycerol also increased oxygen consumption by 80 to 90 and by 60 to $70 \mu\text{l O}_2/\text{h/mg dry wt}$, respectively. Addition of 0.3 % (w/v) Casamino acids increased the oxygen consumption to 220 to $240 \mu\text{l O}_2/\text{h/mg dry wt}$, more than did carbohydrates. The oxygen consumption of the bacterium with individual amino acids was examined at pH 10.0. Alanine caused most active oxygen consumption (190 to $200 \mu\text{l O}_2/\text{h/mg dry wt}$) while serine, glutamic acid and threonine (120 to 130 , 80 to 90 and 80 to $90 \mu\text{l O}_2/\text{h/mg dry wt}$, respectively) were less effective. Little or no oxygen consumption was observed with other amino acids, including proline, valine, leucine, glycine, aspartic acid, histidine, arginine,

Fig. 5

Fig. 6

Fig. 5. Oxygen consumption of an alkalophilic bacterium with carbohydrates at different pH values. ○, Glucose; ●, fructose; △, glycerol.

Fig. 6. Uptake of (○) ^{14}C -labelled AIB or (●), $[\text{U-}^{14}\text{C}]$ glucose into an alkalophilic bacterium at different pH values.

methionine, tryptophan, phenylalanine and lysine. The oxygen consumption of the bacterium on addition of glucose or alanine was 60 and 80% inhibited respectively, by 10^{-2} M-KCN and 90% inhibited by 10^{-2} M-amytal. This suggests that the observed oxygen consumption of the bacterium on addition of carbohydrates or amino acids may be mainly due to electron transport from these substances.

When alanine, serine or glutamic acid (or glucose, or fructose) was added to the extract of the bacteria, oxygen consumption was observed only with alanine. Part of the oxygen consumption of intact bacteria observed with alanine may be due to alanine oxidase. The extract exhibited alanine dehydrogenase activity. The oxygen consumption of intact organisms, when alanine or glutamic acid was used as substrate, may be due to electron transport from the keto acids derived from the amino acids by L-amino acid dehydrogenase (or individual amino acid dehydrogenases). The alanine oxidation (alanine oxidase) and dehydrogenation (alanine or L-amino acid dehydrogenase) activities of the bacterium were both found in the soluble fraction, and the activities were maximum at pH 8 ($8 \mu\text{l O}_2/\text{h/mg}$ protein and $1.1 \mu\text{mol NADH oxidized/min/mg}$ protein, respectively). The enzymes exhibited no activity at pH 10. The results may also suggest the alkalophilicity of the membrane function.

The bacterium exhibited a maximum oxygen consumption at pH 9 to 10 in the presence of either Casamino acids, alanine, serine or glutamic acid (Fig. 4). The rate of oxygen consumption of the bacterium on addition of glucose, fructose or glycerol, on the other hand, did not vary in the pH 7 to 11 region (Fig. 5). The oxygen consumption of the bacterium on addition of pyruvate at pH 10.0 (60 to $80 \mu\text{l O}_2/\text{h/mg}$ dry wt) was higher than that at pH 7.0 (20 to $30 \mu\text{l O}_2/\text{h/mg}$ dry wt). These results suggest that the alkalophilic properties of the bacterium may be related not only to the function of the membrane, but also to the charges of the substances that serve as substrates for biological reactions after entering the organisms across the membrane. The permease enzymes for amino acids (and uracil) may require high pH values.

The effect of pH on the transport of substances into bacteria

The rate of uptake of ^{14}C -labelled AIB into organisms at pH 7.5 or 10.0 was almost linear for about 7 min of incubation. The rates of AIB uptake at different pH values were therefore measured by the radioactivity incorporated into organisms incubated for 5 min. The uptake of AIB was maximum at about pH 10 (Fig. 6), as was the protein synthesis from amino acids and oxygen consumption with amino acids as substrates (Figs. 1 and 4). The uptake of $[\text{U-}^{14}\text{C}]$ glucose into bacteria at pH 7.5 or 10.0 proceeded at a linear rate for about 1 min of incubation. The glucose uptake at different pH values were therefore measured after 1 min incubation. The rates of glucose uptake by the bacterium were unchanged in the pH region 8 to 10, although the rate was slightly less at pH 7 or 11 than that at pH 8 to 10 (Fig. 6). Such a non-alkalophilic property of the bacterium had also been observed for oxygen consumption of the organism on addition of carbohydrates (Fig. 5). Possibly the bacterium exhibits the alkalophilic property only when charged substances cross the membrane.

The oxygen consumption of the bacterium with glucose or Casamino acids at pH 10.5 was saturated at 0.1 M or at 0.3 % (w/v), respectively. When 0.1 M-glucose and 0.3 % (w/v) Casamino acids were added together to the suspension of organisms (pH 10.5), the oxygen consumption ($290\ \mu\text{l O}_2/\text{h/mg}$ dry wt organisms) was the sum of the values with glucose ($95\ \mu\text{l O}_2/\text{h/mg}$ dry wt) and with Casamino acids ($200\ \mu\text{l O}_2/\text{h/mg}$ dry wt). This suggests that the entrance of glucose and amino acids may take place independently.

Alkalostable property of the isolate

Oxygen consumption by suspension of organisms in 0.1 M-phosphate buffer containing 0.9 % (w/v) NaCl was measured at pH 10.0 (37 °C) without added glucose or Casamino acids. In this case, oxygen consumption was linear for at least 15 to 20 min following about 10 min of pre-incubation for temperature equilibration. This suggests that the oxidation system may be stable at pH 10.0 for at least 30 min under these conditions.

When the suspension in phosphate buffer ($E_{650} = 10$) was incubated at pH 7.5 or 10.0 for 3 h at 37 °C, the oxygen consumption with Casamino acids had decreased to 70 % or to 20 % of the original activity, respectively. When the bacterium was incubated at pH 10.0 for 3 h with 0.1 M-glucose or 30 mM-alanine, however, the activity only decreased to 70 or 90 %, respectively. As suggested for an acidophilic thermophilic bacterium (Yamazaki, Koyama & Nosoh, 1973), the alkalophilic bacterium may also have an energy-dependent mechanism for an OH^- exclusion mechanism functioning on the membrane. An investigation with such an approach is now being attempted.

DISCUSSION

Many cultures of alkalophilic bacteria related to the genus *Bacillus* have recently been isolated, and a variety of exoenzymes has been purified from the bacteria (Horikoshi, 1971*a, b*, 1972; Horikoshi & Atsukawa, 1973*a, b*; Kurono & Horikoshi, 1973; Boyer & Ingle, 1972). Since the bacteria are grown in alkaline culture media (pH 10 to 11), it is not surprising that the exoenzymes obtained from the cultures exhibit alkalophilic and alkalostable properties. Alkalophilic bacteria, including our isolate, exhibited maximum growth in alkaline media (pH 10 to 11), and biological activities such as the oxygen consumption with amino acids and the protein and nucleic acid syntheses, examined with intact organisms of our isolate, exhibited maximum activities in alkaline environments (pH 9.0 to 10.5) (Figs. 1, 2 and 4). The results, however, do not indicate that the biological reactions, including those shown in the Figures, will take place in organisms most actively in this pH region. As

deduced from our results (Fig. 3), the intracellular environment of the bacterium may be neutral and the biological reactions occurring internally may exhibit maximum activities in neutral pH. Alanine oxidase and dehydrogenase were located in the soluble fraction of the bacteria, and the enzyme activities were maximum at pH 8 and no activity was observed at pH 10. The alkalophilic property of the oxygen consumption of the bacterium with alanine as substrate, and probably the alkalophilic properties of the organism, in general, may therefore depend on the membrane function of the organisms. The entrance of substances into the bacterium across the membrane (the permease systems) may be considered to be alkalophilic. As shown in Fig. 6, however, not all of the biological reactions of intact bacteria appeared alkalophilic, i.e. the membrane of the bacterium did not seem alkalophilic to all the substances admitted across the membrane. The results (Fig. 6) suggest that the membrane of the bacterium may exhibit the alkalophilic property only when charged substances such as amino acids cross the membrane, but may not show the property in the case of non-charged substances such as glucose, fructose and glycerol.

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