Reisolation and growth conditions of *Bacillus agar-exedens*

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Several agarolytic *Bacillus* strains have been isolated. Their properties agree with those described by Wieringa (1941) for *Bacillus agar-exedens*. These strains are the first reisolates since the original cultures were lost. A second group of isolates is related to the agarolytic *B. palustris* var. *gelaticus* of Sickles and Shaw (1934).

B. agar-exedens requires carbohydrates for growth. In mineral–glucose media growth is inhibited by peptone at pH values of about 7 or less. Under alkaline conditions no inhibition by peptone is observed. A method for the enrichment of *B. agar-exedens* is described.

INTRODUCTION

The existence of aerobic sporeforming bacteria which are able to decompose agar was first reported by Sickles and Shaw (1934), who isolated a single strain which was named *Bacillus palustris* var. *gelaticus*. In 1941 Wieringa described different types of agarolytic *Bacillus* strains, isolated from stable manure, leaf-mould and soils. The type most frequently isolated was described as *B. agar-exedens*. According to Wieringa the main difference between this species and the other strains of agar-decomposing sporeformers was the inability of *B. agar-exedens* to grow in peptone broth. Peptone inhibited growth even when added at low concentrations to a mineral–glucose medium. Growth in the presence of peptone, however, was possible if urea (0.1 %) was present. Allen (1953) reported the isolation of thermophilic, agar-decomposing *Bacillus* strains which were related to *B. stearothermophilus*.

While the strain of *B. palustris* var. *gelaticus* of Sickles and Shaw, now classified as *B. circulans* (Gordon, Haynes and Hor-Nay Pang, 1973) is still available (ATCC 14176, DSM 34), the isolates of Wieringa have been lost

(Wieringa, personal communication). Whether the thermophilic strains isolated by Allen are still available is not known to us.

Since 1968 we have isolated with some difficulty several mesophilic agar-decomposing *Bacillus* strains with the enrichment technique of Wieringa. Studies have been undertaken to find out whether the newly isolated strains are identical with *B. agar-exedens* of Wieringa, with the other unnamed types described by him or with *B. palustris* var. *gelaticus* of Sickles and Shaw. Our studies led to a simple and effective method for the isolation of agar-decomposing *Bacillus* strains.

MATERIALS AND METHODS

Organisms. With the exception of *Bacillus palustris* var. *gelaticus* all strains have been isolated by the method of Wieringa (1941). Soil samples were used for inoculation.

Stock cultures were grown at 30C for 2–3 days on agar slants, consisting of 15 g Bacto-Agar (Difco), 0.005 g $MnSO_4 \cdot H_2O$, 8 g nutrient broth (Difco), 1 g urea in 1000 ml distilled water.

Mineral-glucose medium. The medium contained 1 g $K_2HPO_4 \cdot 3\ H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2\ H_2O$, 0.01 g $FeSO_4 \cdot 7\ H_2O$, 1 g NH_4Cl, 0.005 g $MnSO_4 \cdot H_2O$, 1000 ml distilled water and 10 g glucose. The heat-sterilized glucose solution was added after sterilization. For strains with a requirement for growth factors the medium was supplemented with 0.01 % yeast extract (Difco) or with biotin (1 ng/ml) and/or thiamine (500 ng/ml) sterilized by filtration. The pH of the medium was adjusted after sterilization with NaOH or HCl in the range of pH 6.2 to 7.9. For growth studies aliquots of 30 ml medium were incubated in 300 ml Erlenmeyer flasks at 30C on a rotary shaker (150 rpm).

Peptone broth. The medium consisted of 8 g nutrient broth (Difco), 0.005 g $MnSO_4 \cdot H_2O$, 1000 ml distilled water with or without 10 g glucose. After sterilization (15 min, 120C) the pH was adjusted with NaOH or HCl in the range of pH 6.7 to 8.6.

Physiological characteristics. To test indole production, nitrate reduction and casein decomposition the methods of Smith, Gordon and Clark (1952) were used. The test media for indole production and nitrate reduction were supplemented with 1 % glucose. Anaerobic growth and gas formation from glucose was observed in a urea–peptone broth containing glucose (8 g nutrient broth, 10 g glucose, 1 g urea, 1000 ml distilled water) covered with sterile molten vaspar (equal parts of vaseline and paraffin).

Liquefaction of gelatin was tested in stab cultures of nutrient gelatin (Gelatin, 200 g; Bacto Nutrient Broth, 8 g; distilled water, 1000 ml) after incubation for 2 weeks.

Utilization of and acid formation from starch, agar, glucose, galactose,

sucrose, maltose and lactose were tested with a mineral medium containing 0.5% carbohydrate. To test for hydrolysis of cellulose a sector of filter paper was placed in the tubes. All carbohydrates were sterilized separately from the mineral medium. Agar, starch and cellulose were heat-sterilized (120C, 15 min). All other carbohydrates were sterilized by filtration. For strains with a requirement for growth factors the mineral medium was supplemented with 0.01% yeast extract (Difco) or, if necessary, with 0.5% nutrient broth (Difco). Cultures were incubated in a slant position (5 ml medium per tube). After 6 days the cultures were examined for growth and for acid formation (pH 6.0 or below) by measuring the pH. The pH of all media was adjusted to 7.8. All cultures were incubated at 30C.

Gram staining was carried out according to the standardized method of Bartholomew (1962).

RESULTS

Properties of the new isolates. Applying the original method of Wieringa (1941) we have isolated a total of 24 agarolytic *Bacillus* strains from different soils. All strains formed a visible depression in the surface of agar plates (Fig. 1). In no case, however, true liquefaction of agar was found. This is in accordance

Fig. 1. Colonies of *Bacillus agar-exedens* on peptone agar containing 0.1% urea.

Table 1. Comparison of the properties of *Bacillus agar-exedens* and other agarolytic *Bacillus* strains with newly isolated strains and *B. palustris* var. *gelaticus*.

	Strains isolated by Wieringa		24 newly isolated strains and *Bacillus palustris* var. *gelaticus*			
	B. agar-exedens[1] 6 strains	Unnamed[1] 2 strains	Group I 20 strains	Group II 3 strains	*B. palustris* var. *gelaticus*	Group III 1 strain
Growth in peptone broth	6−	2+	20−	3+	1+	1−
Growth in mineral–glucose medium	6+	1−, 1?	14+[2], 6−	3+[2]	1+[2]	1+[2]
Inhibition by peptone in mineral–glucose medium	6+	1−, 1?	20+	3−	1−	1−
Anti-inhibitory effect of urea	6+		20+			
Rods	6+	2+	20+	3+	1+	1+
Motile	6+	2+	20+	3+	1+	1+
Spores oval	6+	1+, 1−	20+	3+	1+	1+
Sporangium swollen[4]	6+ (?)	?	18−, 2+	3−	1+	1−
Gram reaction positive	6+	2+	20+[3]	3+[3]	1+[3]	1+[3]
Anaerobic growth	6−	2−	20−	3−	1−	1−
Nitrate reduction	6−	2−	20−	3−	1−	1−
Casein decomposition	6−	2−	20−	3−	1−	1−
Indole production	6−	2−	20−	3−	1−	1−
Liquefaction of gelatin	6−	2−	20−	3−	1−	1−
Gas formation from glucose	6−	2−	20−	3−	1−	1−
Starch hydrolysis	6+	2+	8+, 12−	3+	1+	1+
Cellulose hydrolysis	6−	2−	20−	3−	1−	1−
Acid from...						
agar	5+, 1−	2+	20+	3+	1+	1+
glucose	6+	2+	20+	3+	1+	1+
galactose	6+	2+	14+, 6−	3+	1+	1+
sucrose	6+	2+	20+	3+	1+	1+
maltose	6+	2+	18+, 2−	3+	1+	1+
lactose	6+	2+	18+, 2−	3+	1+	1+

[1] Data extracted from Wieringa (1941). [2] Medium supplemented with 0.01 % yeast extract. [3] Young cultures (7 hours) were either gram-positive or stained unevenly. After 36 hours all strains were gram-negative. [4] —: less than 50% of sporangia swollen, +: more than 50% of sporangia swollen.

with Wieringa who observed only a softening of agar around the colonies of his cultures.

The properties of the new isolates as well as of *Bacillus palustris* var. *gelaticus* have been compared with the data given by Wieringa for his 6 strains of *B. agar-exedens* and for his two unnamed cultures (Table 1). The new isolates constitute 3 groups with respect to inhibition by, as well as utilization of, peptone as a carbon source.

The group I strains, to which most of our isolates belong, have been found to be identical with those of Wieringa's strains of *B. agar-exedens* in nearly all properties. The growth factor requirements differed slightly. Fourteen strains of group I showed good growth in a mineral–glucose medium plus 0.01 % yeast extract which could be replaced by biotin and/or thiamin at an initial pH of 6.9. The addition of peptone or nutrient broth to this medium resulted in a strong or complete inhibition of growth. This inhibition was relieved by an additional supplement of 0.1 % urea.

Six other strains of this group apparently have complex growth factor requirements. They were not able to grow in mineral–glucose medium supplemented with 0.01 % yeast extract, also in the presence of urea. Growth was only observed when the mineral–glucose medium was supplemented with 0.5 % peptone or nutrient broth together with urea.

None of the group I strains were able to use peptone as a source of carbon. In urea-containing nutrient broth growth occurred only when glucose or other carbohydrates were present.

Although inhibited by peptone, cultures of group I strains developed slowly on nutrient agar even without urea. However, only a fraction of cells (10^{-2} to 10^{-6}) transferred onto nutrient agar were able to form colonies after prolonged incubation. The same observation has been made by Wieringa who found that with *B. agar-exedens* "in three weeks a small number of colonies may develop on Liebig bouillon agar plates, when these have been sown with a heavy suspension."

The strains of group II differ from group I mainly in two properties: They were not inhibited by peptone in slightly acid or neutral media and did not need carbohydrates for growth. Nutrient broth without urea was a suitable medium for these strains. This group is related to Wieringa's strain 8.62 and also includes *B. palustris* var. *gelaticus*.

Group III contains only a single isolate which is possibly an intermediate between groups I and II. This strain was not inhibited by peptone but needed carbohydrates for growth.

Growth inhibition by peptone and influence of pH values. Neither the inhibition of *B. agar-exedens* by peptone nor its neutralization by urea were explained by Wieringa. In the present study the mechanism of peptone inhibition was not investigated. We have found, however, that the anti-inhibitory effect of urea was merely an effect of pH. During heat-sterilization of urea-containing

nutrient broth the pH of the medium was raised from pH 6.9 to about 7.8. Upon addition of glucose, this medium was excellent for growing our group I strains. If urea was omitted and the pH of the medium after sterilization was adjusted to pH 7.8 with sodium hydroxide the same good growth as in the presence of urea was observed.

The pH-dependent growth of group I strains in mineral–glucose medium (plus 0.01 % yeast extract, sometimes replaced by biotin and/or thiamine) and in nutrient broth plus glucose is illustrated in Figs. 2 and 3. Whereas in mineral–glucose medium growth occurred already at an initial pH of 6.4 (optimum at pH 6.9 and higher), growth of the same strain in nutrient broth plus glucose was observed only above pH 7.0 (optimum above pH 7.9). The minimum pH values allowing growth in the presence of peptone vary from strain to strain and have also been found to be dependent on the type of inoculum as well as on the concentration of nutrient broth or peptone. With decreasing concentrations of the latter compounds in mineral–glucose medium pH values allowing good growth continuously dropped to the neutral range. No such differences with respect to the pH values of media have been observed with the strains of our groups II

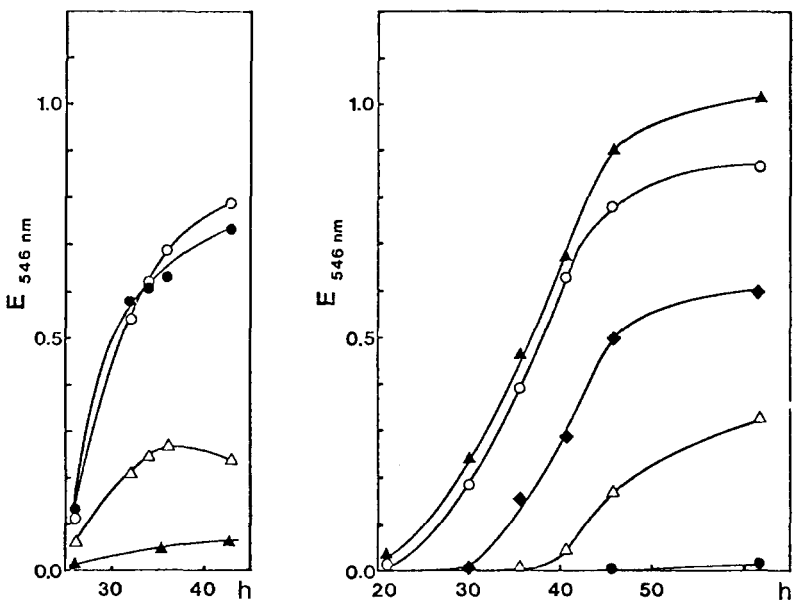

Fig. 2. Growth ($E_{546\,nm}$) of a strain from group I (*33b*) in mineral–glucose medium supplemented with 1 ng biotin/ml at different pH values.
pH 6.2▲——▲, pH 6.4 △——△, pH 6.9 ○——○, pH 7.9 ●——●

Fig. 3. Growth ($E_{546\,nm}$) of a strain from group I (*33b*) in peptone broth with 1 % glucose at different pH values.
pH 6.9 ●——●, pH 7.1 △——△, pH 7.4◆——◆, pH 7.9 ○——○, pH 8.6▲——▲.

and III. At pH 6.7 no growth inhibition by peptone could be observed (Fig. 4) although final turbidities of the cultures have been found to be higher at starting pH values of 7.2 or higher.

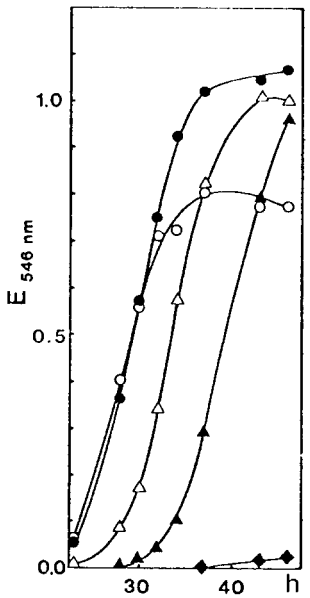

Fig. 4. Growth ($E_{546\,nm}$) of a strain from group II (*Bacillus palustris* var. *gelaticus*) in peptone broth with 1% glucose at different pH values.
pH 6.7 ○——○, pH 7.2 ●——●, pH 7.6 △——△, pH 8.0 ▲——▲, pH 8.3 ◆——

Enrichment of agarolytic sporeforming bacteria. Applying Wieringa's enrichment technique we detected agarolytic sporeformers in only a few of many different soil samples. Van der Meulen, Harder and Veldkamp (1974) failed to isolate such organisms. These results are contradictory to Wieringa's observations who stated that "agar decomposing bacilli are relatively common". Very often our enrichment cultures contained less than 100 agarolytic cells in 1 ml, estimated by colony formation on mineral–agar plates; thus they often could not be detected simply by streaking a loopful of the enrichment culture on plates.

After we had found that even small pH changes may very strongly affect the growth of *B. agar-exedens* in the presence of organic matter, we assumed that unfavourable pH values of enrichment cultures may be responsible for the discrepancies mentioned. Therefore we modified Wieringa's method in the following way: 2.5 g of soil, rich in organic matter, were suspended in 10 ml sterile water and pasteurized at 80C for 10 min. Two ml of the soil suspension were transferred to a 300-ml Erlenmeyer flask containing 30 ml mineral–

medium supplemented with 0.1 % (w/v) Bacto-Agar as a source of carbon. The pH of the medium was brought to 7.9–8.2 with sodium hydroxide, and the enrichment cultures were incubated at 30C for 5 to 6 days on a rotary shaker (150 rpm).

In such cultures agarolytic bacteria grew to 10^5 to 10^7 cells/ml. They were readily isolated by streaking a loopful of the suspension on mineral–agar plates, supplemented with 0.01 % yeast extract and adjusted to pH 7.8. Agar-decomposing colonies were easily detected, due to the formation of depressions on the agar surface; they were purified by streaking on nutrient agar plates (pH 8.0). To select for *B. agar-exedens* (group I strains) the isolated cultures had to be transferred to nutrient broth (pH 6.9) and to nutrient broth plus 1 % glucose (pH 8.0). Strains which grew only in the glucose-containing medium were presumably *B. agar-exedens*.

DISCUSSION

Since the strains of our group I correspond in almost all properties with those of *Bacillus agar-exedens* as described by Wieringa (1941), we consider our strains to be new isolates of this "lost" species.

Most important — in addition to the agarolytic property, which apparently is rare among *Bacillus* species — is the growth inhibition by peptone and its reversal under alkaline conditions. This inhibitory effect of peptone has not been observed with any other known *Bacillus* species.

In spite of this similarity some differences between the properties of our strains and those of *B. agar-exedens* as described by Wieringa should be discussed. In contrast to the observation of Wieringa, all strains of group I have a requirement for vitamins, expecially biotin and/or thiamine, or for unknown compounds present in peptone. These differences, however, should not lead to a separation of these strains from *B. agar-exedens*. The tap water used by Wieringa to prepare his media may have contained vitamins in the same small amounts as needed by some of our group I strains. Glucose may have been contaminated by growth factors, too. On the other hand, requirements for vitamins or amino acids may vary substantially between different strains of a certain *Bacillus* species (Knight and Proom, 1950; Koser, 1968; White, 1972). Also the differences observed with some strains in respect to acid formation from some sugars and to hydrolysis of starch do not justify a separation of these strains from *B. agar-exedens* at present. This question will be further studied by DNA-DNA hybridization experiments.

In addition to the properties of *B. agar-exedens*, as described by Wieringa, we have found that all strains inhibited by peptone need carbohydates for growth. This dependence was not examined by Wieringa.

Wieringa has found that during sporulation of his strains "clostridial forms

are common". Possibly this agrees with our observations, that in most cultures swollen, sporulated cells are formed in addition to unswollen sporangia. A similar mixture of swollen und unswollen forms within a certain culture was found by Bonde (1973) also in other *Bacillus* species.

Like Wieringa we have isolated some agarolytic spore-formers (our groups II and III) which are not inhibited by peptone and which are not classified as *B. agar-exedens* by Wieringa. With the exception of one strain (group III) these strains do not need carbohydrates for growth as do the group I strains. The strains have properties in common with *B. palustris* var. *gelaticus*, which now is considered to be a strain of the heterogenous *B. circulans* complex (Gordon et al., 1973).

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