UREA-HYDROLYZING BACILLI

II. Nutritional Profiles

G. H. BORNSIDE[1] AND R. E. KALLIO

Department of Bacteriology, College of Medicine, State University of Iowa, Iowa City, Iowa

Received for publication October 18, 1955

Knight and Proom (1950) surveyed the nutritional requirements of some 200 strains of mesophilic bacilli. Their data indicated a degree of uniformity of specific nutritional patterns allowing preliminary allocation of new isolates to their probable species by nutritional screening. They appraised their results as "a striking confirmation of the validity" of the classification of Smith and co-workers (1946).

The primary objective of the present report was to ascertain the extent to which nutritional requirements are characteristic of species of the genus *Bacillus* as differentiated by the physiological properties used in the preliminary identification of urea-hydrolyzing bacilli isolated from urea-enrichment cultures (Bornside and Kallio, 1956). The results reported extend and further substantiate our previous findings.

MATERIALS AND METHODS

Organisms used were from the collections of bacilli previously studied (Bornside and Kallio, 1956).

Medium. The casein basal solution employed contained vitamin-free casein hydrolyzate (Nutritional Biochemical Corp.; either acid or enzyme hydrolyzed), 0.5 per cent final concentration; L-cystine, 10 mg; DL-tryptophan, 100 mg; double strength mineral base solution without added NH_4^+ (Hunter *et al.*, 1950), 250 ml; distilled water to 800 ml. The pH of this basal solution was adjusted to 9.0 with NaOH. The solution was then boiled and filtered. To 250-ml Erlenmeyer flasks, or special growth flasks (to be described below), were added 40 ml of basal solution. The volume in each vessel was brought to 50 ml by addition of *tris*-(hydroxymethyl)-aminomethane buffer, vitamins, and urea.

Thiamin and pantothenic acid were sterilized by passage through UF-sintered glass filters, and added aseptically to the autoclaved basal medium. Urea was added to the sterile medium in the form of a 10 M solution, which had undergone "autosterilization" for several days before using. Solutions of vitamins 50 times the final concentration were employed. Final concentrations of the several supplements to the casein basal solution were as follows: urea, 0.2 M; biotin, 1 mμg per ml; thiamin-HCL, 0.5 μg per ml; nicotinic acid, 0.5 μg per ml; pantothenic acid, 0.5 μg per ml; "tris" buffer (pH 9.5), 0.1 M.

Growth vessels. Growth was studied at room temperatures (25 to 30 C) using 50-ml cultures in 250-ml flasks agitated on a rotary shaker (New Brunswick Scientific Co.). Specially modified culture flasks, similar in principle to those described by Wiame and Storck (1953), were employed consisting of 250-ml Erlenmeyer flasks from the outer wall of which a short curved side-arm projected upward, the mouth of the flask being sealed with a bored rubber stopper into which was fitted an inverted 10-ml Klett tube. The side-arm, fitted with a cotton plug, allowed introduction of the inoculum and sterile supplements to the medium. Basal medium was autoclaved in the complete assembly. The culture was incubated upon the shaker, and growth was followed turbidimetrically as a function of time. For measurement of turbidity, the inverted flask was inserted in a Klett-Summerson colorimeter (blue filter) by means of the Klett tube. Thus growth was studied in an ostensibly closed system; samples did not have to be withdrawn for measurement of turbidity, and the danger of contamination was minimized.

Turbidity was expressed in terms of Klett readings, and was measured immediately after inoculation and at intervals until the stationary phase of growth was achieved.

Inoculum. An 18-hr slant culture was suspended in 1 ml of sterile distilled water, and inoculated into 50 ml of the appropriate complete medium (either with or without added urea).

[1] Present address: Department of Bacteriology, University of Georgia, Athens, Georgia.

TABLE 1

Nutritional requirements of urea-hydrolyzing bacilli

	Strain	No. of Strains	Nutrients Required					
			Amino acids	Ammonia from urea	Thiamin	Biotin	Nicotinic acid	Pantothenic acid
	Steno-responsive							
B. pasteurii:	*	2	+†	−‡	+	+	−	
	*	2	+	−	+	−	+	
	*	4	+	+	+	−	+	
	NRS 929		+	+	±§	−	+	
	NRS 673		+	+	+	−	+	
	U/A-15		+	+	+	−	±	
	*	2	+	+	+	+	−	
	93		+	+	+	±	−	
	U-22		+	+	+	+	−	
	85		+	+	+	−	−	
	U/A-24; U/A-37	2	+	+	+	±	+	
	U/B-7		+	+	+	+	+	
	89; Gibson 22; NRS 674; NRS 675	4	Unidentified; no growth on complete medium					
	Eury-responsive; ureolytic							
B. freudenreichii:	NRS 671; R-18; Q-27; R-83	4	+	−	−	−	−	
Intermediate:	NRS T-156		+	−	±	−	−	
	NRS 1023; S-9	2	+	−	+	−	−	
	NRS 339; T-16; S-91	3	+	−	+	±	−	
	R-3		+	−	−	±	−	
	R-19; 81	2	+	−	−	+	−	
	R-28		+	−	−	±	+	
	NRS 350; NRS 866	2	Unidentified; (due to granular growth)					
B. loehnisii:	NRS 672		+	−	±	±	+	
	R-20; 92	2	+	−	+	±	+	
	R-17		+	−	+	±	±	
	T-2; 13	2	+	−	+	+	+	
B. lentus:	82; NRS 670; NRS 1262; NRS 883	4	Unidentified; complex; no growth					
	Eury-responsive; nonureolytic							
B. sphaericus:	12		+	−	−	+	−	
	NRS 967		−	−	−	−	−	
	NRS 966		+	−	±	−	−	
	NRS 810		+	−	+	−	−	
	NRS 719		+	−	±	±	−	
	NRS 348		+	−	±	+	−	
B. pantothenticus:	NRS 1317; NRS 1318; NRS 1319	3	+	−	+	±	−	+
	NRS 1320; NRS 1321	2	+	−	+	±	−	±
	NRS 1322		+	−	±	±	−	±

* Results reported by Knight and Proom (1950).

† Essential.

‡ Stimulatory.

§ Not required.

After this culture, in a 250-ml Erlenmeyer flask, was incubated on the shaker for 24 hours, 5 ml were transferred to a second flask of the same medium. Growth from this second culture (and in some cases from a third culture) was centrifuged, washed three times in sterile saline, and resuspended in 1 ml of saline. Each growth vessel was then inoculated with 0.1 ml of this washed suspension.

RESULTS

Steno-responsive, ureolytic bacilli. Four of the 13 strains of *B. pasteurii* examined would not grow in the complete experimental medium, and their growth requirements remain to be elucidated. Results of the growth-factor study are recorded in table 1, along with data presented by Knight and Proom (1950). We concur with these workers in that the nutritional requirements of *B. pasteurii* strains are heterogeneous. Nine of our strains required amino acids, ammonia, and thiamin for growth. Contrary to the results of Knight and Proom, no correlation was found between the requirement for either nicotinic acid or biotin. Although Knight and Proom noted that *B. pasteurii* strain NRS no. 674 might possibly have required both biotin and nicotinic acid in addition to amino acids, ammonia, and thiamin, the experimental conditions employed by us did not permit the growth of strain NRS 674. However, 3 of 9 strains clearly show a requirement for both biotin and nicotinic acid; one strain required neither biotin nor nicotinic acid for growth. None of our strains of *B. pasteurii* grew unless free ammonia, resulting from the hydrolysis of added urea, was in the medium. Lack of agreement with the findings of Knight and Proom is apparently a consequence of the rigorous physiological criteria used to define steno-responsive, ureolytic bacilli (Bornside and Kallio, 1956). Figures 1 and 2 are representative plots of the various nutritional patterns exhibited by *B. pasteurii*. These growth curves emphasize the essential requirement for free ammonia which is characteristic for each strain of *B. pasteurii*, and is reflected in the physiological characterization of steno-responsive ureolytic bacilli.

Eury-responsive, ureolytic bacilli. Although physiological study of each of the known strains of *B. freudenreichii* and *B. loehnisii* available from the Smith collection showed no qualitative difference between the two species (Bornside and Kallio, 1956), investigation of the nutritional requirements of these two organisms (table 1) indicated that *B. freudenreichii* NRS 671 required only amino acids for growth. On the other hand, *B. loehnisii* NRS 672 not only required amino acids and nicotinic acid, but was stimulated by thiamin and biotin. These distinct nutritional profiles seem to allow the recognition of the following previously unidentified organisms as strains of *B. freudenreichii:* strains R-18, Q-27, and R-83. Likewise strains T-2, R-20, 13, and 92 have been recognized as *B. loehnisii*. The nicotinic acid requirement exhibited by all strains of *B. loehnisii* and an intermediate strain is noteworthy in light of the report by Knight and Proom (1950) that some strains of *B. pasteurii* exhibited a requirement for nicotinic acid. Figures 3 and 4 are plots of growth curves for representative strains of *B. freudenreichii* and *B. loehnisii*. In addition to anchoring the spectrum of nutritional profiles displayed by eury-responsive ureolytic bacilli, these figures illustrate the precision of the data summarized in table 1.

The complete casein-vitamin-urea medium employed failed to support the growth of *B. lentus* strain 82 and the three strains of *B. lentus* from the Smith collection. The complete medium supplemented with pantothenic acid, riboflavin, *p*-aminobenzoic acid, and pyridoxal

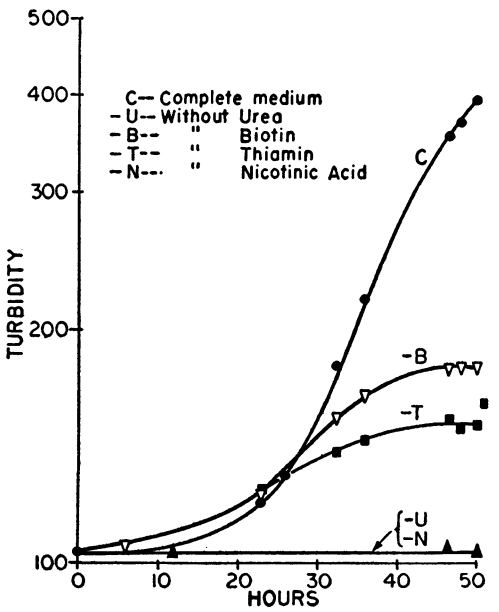

Figure 1. Growth curves for *B. pasteurii* strain U/B-7 in a casein hydrolyzate-basal medium supplemented with urea, biotin and nicotinic acid.

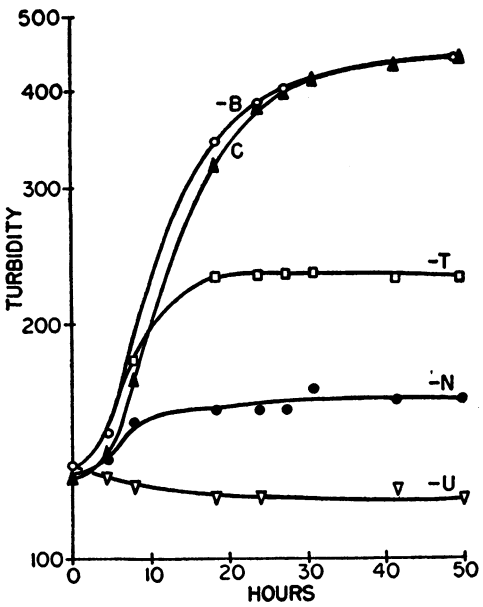

Figure 2. Growth curves for *B. pasteurii* NRS strain 929 in a casein hydrolyzate-basal medium supplemented with urea, biotin, thiamin, and nicotinic acid; *C* = complete medium; $-B$ = minus biotin; $-T$ = minus thiamin; $-N$ = minus nicotinic acid; $-U$ = minus urea.

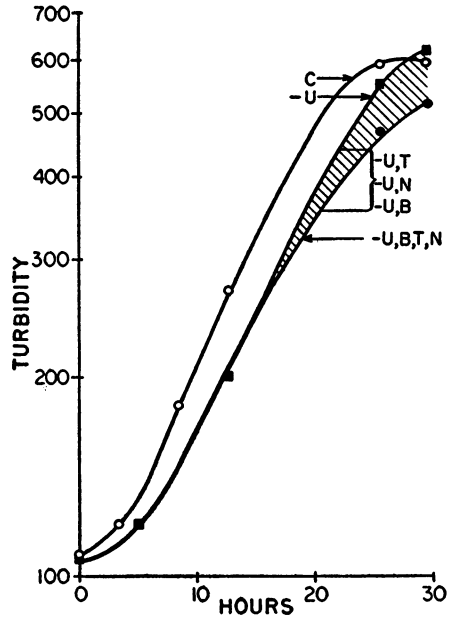

Figure 3. Growth curves for *B. freudenreichii* NRS strain 671 in a casein hydrolyzate-basal medium supplemented with urea, biotin, thiamin, and nicotinic acid. *C* = complete medium; $-U$ = minus urea; $-U,T$ = minus urea and thiamin; $-U,N$ = minus urea and nicotinic acid; $-U,B$ = minus urea and biotin; $-U,B,T,N$ = minus urea, biotin, thiamin, and nicotinic acid.

did not support growth of the three NRS strains of *B. lentus*, but did allow strain 82 to grow slightly. The nutritional requirements of *B. lentus* were not investigated further. The results of these preliminary experiments, however, confirm the experience of Knight and Proom who also found complex nutritional requirements for organisms in the *B. firmus-B. lentus* group. *B. firmus* and *B. lentus*, which Smith and co-workers (1952) assign to morphological group 1, differ in that the latter exhibits urease activity and hydrolyzes neither gelatin nor casein. Gibson (1935) considered *B. lentus* to be sufficiently well defined to justify its recognition as a distinct species related to *B. pasteurii*.

The differentiation between *B. sphaericus* and *B. sphaericus* var. *fusiformis* has been based upon the absence of urease from the former and its presence in the latter (Smith *et al.*, 1946). Knight and Proom (1950) reported that 6 of 9 strains of *B. sphaericus* grew on a casein basal medium with added thiamin, whereas the remaining strains, and 10 strains of *B. sphaericus* var. *fusiformis*, required biotin in addition. Neither thiamin nor biotin was effective singly.

Since no correlation existed between nutritional requirements and ureolytic activity, Smith and co-workers (1952) considered *B. sphaericus* var. *fusiformis* to be a biotype of *B. sphaericus* and the hydrolysis of urea to be a variable characteristic of the species. Apparently the significance of the ability to hydrolyze urea as a key diagnostic criterion in the identification of strains of the genus *Bacillus* has not been fully appreciated. The utility of urease activity as a diagnostic characteristic is, moreover, enhanced by its independence from nutritional requirements, and thus lends itself to use as a primary character in a dichotomous key for the identification of individual strains of the genus *Bacillus*.

Eury-responsive, nonureolytic bacilli. Seven of the 12 known strains of *B. sphaericus* were urease-negative. The nutritional requirements of 5 of these urease-negative strains and of the sole urease-negative isolate from urea enrichments are summarized in table 1. These strains are similar nutritionally to the several intermediate strains in the urease-positive eury-

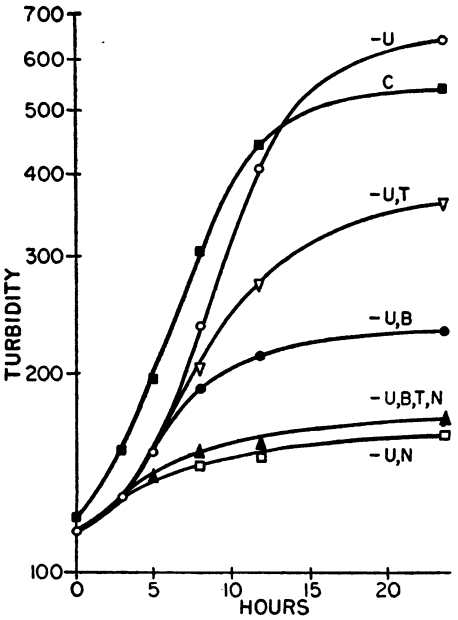

Figure 4. Growth curves for *B. loehnisii* NRS strain 672 in a casein hydrolyzate-basal medium supplemented with urea, biotin, thiamin, and nicotinic acid. *C* = complete medium; $-U$ = minus urea; $-U,T$ = minus urea and thiamin; $-U,B$ = minus urea and biotin; $-U,N$ = minus urea and nicotinic acid; $-U,B,T,N$ = minus urea, biotin, thiamin, and nicotinic acid.

responsive category. The nutritional profile of NRS strain 967 appears aberrant. The complete medium, with or without urea, failed to support the growth of *B. rotans* NRS strain 633.

All 6 strains of *B. pantothenticus* required amino acids, thiamin, biotin, and pantothenic acid. They present an extremely homogeneous nutritional profile (table 1). The characteristics of this species both bacteriologically (Proom and Knight, 1950; Smith et al., 1952) and physiologically (Bornside and Kallio, 1956), are distinctive and unique.

DISCUSSION

The results of this study have allowed the identification of bacilli isolated from 5 per cent urea enrichment cultures. Organisms obtained are urease-positive with the exception of one isolate. *B. pasteurii* appears to be a homogeneous species on the basis of its limited bacteriological and physiological properties, being steno-responsive to pH and requiring free ammonia. These requirements may be met with a medium of pH 8.5–9.0 plus ammonia or by inclusion of urea in the medium. On the more precise level of growth-factor requirements the strains exhibited more complex patterns.

It is suggested that steno-responsive ureolytic bacilli, for which free ammonia in addition to amino acids are essential, be considered as strains of *B. pasteurii*. By this definition, the first two nutritional profiles reported by Knight and Proom and shown in table 1 characterize strains which are not *B. pasteurii*, but conceivably eury-responsive ureolytic bacilli.

A rational physiological approach to identification of urea-hydrolyzing bacilli has also established the existence of a large, homogeneous group of bacilli eury-responsive to pH. On the nutritional level, however, an extreme degree of heterogeneity again exists. Comparison with known species of bacilli identified by Smith and co-workers (1946, 1950) suggested identifications impossible at the physiological level of analysis. It is proposed that eury-responsive

TABLE 2

Provisional key for the identification of several species in the genus Bacillus

I. Urease-positive; require amino acids	
A. Steno-responsive; require NH_3	*B. pasteurii*
B. Eury-responsive; do not require NH_3	
1. Growth at pH 5 to 11	*B. loehnisii*
2. No growth at pH 5; growth at pH 7 to 11 (optimal at pH 7); nutritionally complex	*B. lentus*
II. Urease-negative; require amino acids; eury-responsive; do not require NH_3; do not require nicotinic acid	
A. Growth at pH 5 to 11; require either biotin or thiamin, or both	*B. sphaericus*
B. No growth at pH 5; growth at pH 7 to 11	
1. Require pantothenic acid in addition to thiamin, and biotin	*B. pantothenticus*
2. Nutritionally complex	*B. rotans*

ureolytic bacilli be united in a single species, *B. loehnisii* Gibson, since they possess similar nutritional heterogeneity in regard to vitamin requirements, but are distinctive in not needing free ammonia. This species, related to *B. pasteurii*, was also isolated from urea enrichments. Physiological characterization indicates only slight differences between *B. loehnisii* and *B. lentus* Gibson. The latter (Gibson, 1935) is distinct bacteriologically although its nutritional profile at present is unknown.

Although the nonureolytic, eury-responsive category comprised *B. sphaericus*, *B. rotans*, and *B. pantothenticus*, the latter were not isolated from urea enrichment cultures. The nutritional characteristics of *B. pantothenticus* are homogeneous. The known strains of *B. sphaericus* were physiologically identical, i.e., eury-responsive, nonureolytic, but the nutritional profiles exhibited by the 6 strains examined were heterogeneous. Individual strains appear to require either thiamin or biotin, or both.

There was agreement with Knight and Proom (1950) that nicotinic acid is required by some strains of *B. pasteurii*. Evidence indicating a requirement for nicotinic acid by 7 strains of eury-responsive, ureolytic bacilli is of further interest as Knight and Proom noted that an aberrant strain of *B. brevis* also exhibited a requirement for nicotinic acid. Aside from a single strain of *B. coagulans* and groups of thermophilic bacilli (Cleverdon *et al.*, 1949*a*, 1949*b*), nicotinic acid has not previously been known to be required by species of the genus *Bacillus*.

The dichotomous key in table 2 summarizes the results of this investigation. The key is proposed as an auxiliary aid to the identification of several species, which for the most part belong to morphological group 3 and show natural relationships. Although workable, the key is tentative in regard to nutritionally complex species such as *B. lentus* and *B. rotans*. *B. pantothenticus* is included for the total representation of group 3.

SUMMARY

The vitamin requirements for ureolytic bacilli were generally satisfied by various combinations of nicotinic acid, biotin, and thiamin. In addition to amino acids, free ammonia was also required for the growth of *Bacillus pasteurii*. Nutritional screening allowed identification of the bacilli isolated from soil. Strains of *B. pasteurii* displayed great heterogeneity nutritionally, although physiologically they were all steno-responsive.

Nicotinic acid was shown to be required by several strains of *B. pasteurii* and *Bacillus loehnisii*. A dichotomous key for the identification of several species in the genus *Bacillus* is proposed.

REFERENCES

BORNSIDE, G. H. AND KALLIO, R. E. 1956 Urea-hydrolyzing bacilli. I. A physiological approach to identification. J. Bacteriol., **71**, 627–634.

CLEVERDON, R. C., PELCZAR, M. J., JR., AND DOETSCH, R. N. 1949*a* Vitamin requirements of *Bacillus coagulans*. J. Bacteriol., **58**, 113.

CLEVERDON, R. C., PELCZAR, M. J., JR. AND DOETSCH, R. N. 1949*b* The vitamin requirements of stenothermophilic aerobic sporogenous bacilli. J. Bacteriol., **58**, 523–526.

GIBSON, T. 1935 The urea-decomposing microflora of soils. I. Description and classification of the organisms. Zentr. Bakteriol. Parasitenk., II, **92**, 364–380.

HUTNER, S. H., PROVASOLI, L., SCHATZ, A. AND HASKINS, C. P. 1950 Some approaches to the role of metals in the metabolism of microorganisms. Proc. Am. Phil. Soc., **94**, 152–170.

KNIGHT, B. C. J. G. AND PROOM, H. 1950 A comparative survey of the nutrition and physiology of mesophilic species of the genus *Bacillus*. J. Gen. Microbiol., **4**, 508–538.

PROOM, H. AND KNIGHT, B. C. J. G. 1950 *Bacillus pantothenticus* (n. sp.). J. Gen. Microbiol., **4**, 539–541.

SMITH, N. R., GORDON, R. E. AND CLARK, F. E. 1946 *Aerobic mesophilic sporeforming bacteria*. Misc. Publ. No. 559, U. S. Dept. Agr., Washington, D. C.

SMITH, N. R., GORDON, R. E. AND CLARK, F. E. 1952 *Aerobic sporeforming bacteria*. Agriculture Monograph No. 16, U. S. Dept. Agr., Washington, D. C.

WIAME, J. M. AND STORCK, R. 1953 Metabolisme de l'acide glutamique chez *Bacillus subtilus*. Biochim. et Biophys. Acta., **10**, 394–404.