

NUTRITIONAL REQUIREMENTS OF *BACILLUS ALVEI* AND *BACILLUS PARA-ALVEI*¹

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Received for publication October 7, 1946

Although bacteria associated with foul brood of bees have been studied by many investigators, very little was done with regard to the specific nutritional requirements of these organisms until it was demonstrated (Lochhead, 1942) that *Bacillus larvae*, etiological agent of American foul brood, required thiamine for growth. The studies have been extended at this laboratory to include investigations of the growth requirements of bacteria usually associated with foul brood disease but not necessarily the causal agents. The results of studies on the requirements of two strains of *Streptococcus apis* are presented in another paper (Katznelson, 1947). The present paper deals with *Bacillus alvei*, an organism repeatedly isolated from bee larvae affected with European foul brood but with no clearly established pathogenic properties, and *Bacillus para-alvei*, isolated from larvae affected with "para-foulbrood" disease of bees (Burnside, 1932; Burnside and Foster, 1935). Some question has arisen as to the distinction between this organism and *B. alvei*. Tarr (1936), on comparing their general morphological, cultural, and biochemical characteristics, concluded that the only distinguishing feature was a purely morphological one, involving change in the shape of the vegetative cells during sporulation and in the shape of the spores. Smith *et al.* (1946) considered the two organisms identical. It was hoped, therefore, that a study of the growth requirements of these bacteria might throw some light on their relationship in addition to furnishing information concerning their specific nutritional needs.

EXPERIMENTAL METHODS

Four stock strains of *B. alvei* and one of *B. para-alvei* were available for study at the outset; subsequent tests included six freshly isolated strains of *B. alvei* and two strains of *B. para-alvei*.³

To double strength basal medium containing 5.0 g glucose per liter and inorganic salts (Lochhead, 1942) were added various combinations of nitrogenous materials of varying complexity and vitamins of the B complex, and the solution was diluted to the desired volume. The medium was then adjusted to a pH of 6.8 to 7.0, dispensed in 8-ml amounts, and autoclaved for 15 minutes at 15 pounds' pressure. Inoculum was prepared by suspending cells from a 24-hour nutrient agar culture in 0.9 per cent saline, until a faint turbidity was obtained;

¹ Contribution No. 225 (Journal Series) from the Division of Bacteriology and Dairy Research, Science Service, Department of Agriculture, Ottawa.

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³ Kindly supplied by N. R. Smith, USDA, Beltsville, Md.

one 1-mm loopful was used per tube of medium. Growth was determined visually after 48 and 96 hours' incubation at 37 C. All glassware was scrupulously cleaned (acid-dichromate) and rinsed.

The following chemicals were tested:

Vitamins (μg per liter)

Thiamine.....	200	β -Alanine.....	200
Riboflavin.....	200	<i>para</i> -Aminobenzoic acid.....	200
Pyridoxine.....	200	Biotin.....	0.1
Pantothenic acid.....	200	Folic acid concentrate ⁴	10
Nicotinic acid.....	200	Inositol.....	50 mg

Amino acids (mg per liter)

Glycine.....	80	<i>l</i> -Tryptophane.....	200
<i>dl</i> -Alanine.....	80	<i>l</i> -Cystine.....	250
<i>dl</i> -Valine.....	320	<i>dl</i> -Lysine·2HCl.....	400
<i>l</i> -Leucine.....	100	<i>l</i> -Histidine·HCl.....	160
<i>l</i> -Aspartic acid.....	100	<i>d</i> -Arginine·HCl.....	160
<i>d</i> -Glutamic acid.....	500	<i>l</i> -Proline.....	200
<i>dl</i> -Serine.....	80	<i>dl</i> -Isoleucine.....	200
<i>dl</i> - β -Phenylalanine.....	200	<i>dl</i> -Methionine.....	160
<i>l</i> -Tyrosine.....	140	<i>l</i> -Asparagine.....	500

EXPERIMENTAL RESULTS

Vitamin requirements. An attempt was first made to grow the organisms in media containing different sources of nitrogen with and without the vitamins of the B complex. The results (table 1) indicate that only casein hydrolyzate plus vitamins, or yeast extract, permitted growth of all strains; simpler nitrogenous compounds were ineffective. By omitting each of the 10 vitamins singly or in groups from a mixture of all in a casein hydrolyzate medium, it was found that thiamine was essential for growth. When amino acids were substituted for casein hydrolyzate, similar results were obtained with *B. alvei* strains (table 2). However, it was found repeatedly that *B. para-alvei* grew moderately well in the amino acid mixture alone, but was apparently stimulated by added thiamine (table 3).

Amino acid requirements. Each acid was omitted from a mixture of 18 in the basal medium containing thiamine. None of the acids appeared to be essential, although some were stimulatory at 96 hours. After further work on various combinations of acids the number required to give good growth of all strains was reduced to 14. When each one of these was omitted singly from the mixture, it was found that the omission of glycine, leucine, and cystine reduced growth of *B. alvei* strains appreciably (table 2). The omission of glycine from a medium containing thiamine markedly depressed growth of all three strains of *B. para-alvei* (table 3). In the absence of this vitamin, growth was also reduced when valine, phenylalanine, and isoleucine were left out of the medium, and was almost negligible in tubes devoid of cystine.

⁴ Kindly supplied by R. J. Williams, University of Texas, as a concentrate, "potency 3,100."

Subsequently, 6 fresh strains of *B. alvei* were isolated and tested for thiamine and amino acid requirements. At the same time an attempt was made to sub-

TABLE 1
Growth of Bacillus alvei and Bacillus para-alvei in media containing different sources of nitrogen and B vitamins

ADDENDA TO BASAL MEDIUM	GRAMS PER LITER	B. ALVEI STRAINS				B. PARA-ALVEI
		127	179	343	408	316
(NH ₄) ₂ SO ₄	3.0	-	-	-	-	-
(NH ₄) ₂ SO ₄ + vitamins.....		-	-	-	-	-
KNO ₃	2.0	-	-	-	-	-
KNO ₃ + vitamins.....		-	-	-	-	-
Asparagine.....	1.0	-	-	-	-	-
Asparagine + vitamins.....		-	-	-	-	-
Casein hydrolyzate.....	4.0	-	-	-	-	-
Casein hydrolyzate + vitamins....		+++	++++	+++	+++	+++
Yeast extract.....	4.0	++++	++++	++++	++	++++

++++ = maximum turbidity at 96 hours.

- = no growth.

TABLE 2
Thiamine and amino acid requirements of B. alvei

ADDENDA TO BASAL MEDIUM	STRAINS			
	127	179	343	408
(1) Casein hydrolyzate.....	-	-	-	-
(2) Casein hydrolyzate + thiamine..	+++	+++	+++	+++
(3) 18 amino acids.....	-	-	-	-
(4) 18 amino acids + thiamine.....	+++	+++	+++	+++
(5) 14 amino acids + thiamine.....	+++	+++	++++	+++
(6) (5) - Glycine.....	+	+	+	++
(7) (5) - Alanine.....	++	+++	+++	+++
(8) (5) - Valine.....	++	++	+++	+++
(9) (5) - Leucine.....	+	+	+	+
(10) (5) - Aspartic acid.....	++	++	+++	+++
(11) (5) - Glutamic acid.....	+++	++	+++	++
(12) (5) - Serine.....	+++	++	+++	+++
(13) (5) - Phenylalanine.....	++	++	++++	+++
(14) (5) - Tyrosine.....	+++	++	+++	++++
(15) (5) - Tryptophane.....	++	++	+++	+++
(16) (5) - Cystine.....	+	++	+	-
(17) (5) - Histidine.....	+++	++	++++	+++
(18) (5) - Arginine.....	++	++	++++	+++
(19) (5) - Methionine.....	++	++	++++	++++

stitute (NH₄)₂SO₄ for some of the amino acids in a medium containing glycine, leucine, and cystine. The treatments and results obtained are given in table 4.

TABLE 3
Thiamine and amino acid requirements of *B. para-alvei*

ADDENDA TO BASAL MEDIUM	STRAINS					
	316		551		552	
	+B ₁	-B ₁ *	+B ₁	-B ₁	+B ₁	-B ₁
(1) Casein hydrolyzate.....	+++	-	+++	-	+++	-
(2) 18 Amino acids.....	+++	++	+++	++	+++	++
(3) (1) - Glycine.....	+	±	+	±	+	±
(4) (2) - Alanine.....	+++	++		++		++
(5) (2) - Valine.....	+++	+	+++	+	+++	+
(6) (2) - Leucine.....	+++	+	+++	++	+++	++
(7) (2) - Aspartic acid.....	+++	++		++		++
(8) (2) - Glutamic acid.....	+++	++		++		++
(9) (2) - Serine.....	+++					
(10) (2) - Phenylalanine.....	+++	±	+++	+	+++	+
(11) (2) - Tyrosine.....	+++	++		++		++
(12) (2) - Tryptophane.....	+++	++		++		++
(13) (2) - Cystine.....	+++	-	+++	±	+++	±
(14) (2) - Lysine.....	+++	++		++		++
(15) (2) - Histidine.....	+++	++		++		++
(16) (2) - Arginine.....	+++	++		++		++
(17) (2) - Proline.....	+++					
(18) (2) - Isoleucine.....	+++	+	+++	+	+++	+
(19) (2) - Methionine.....	+++					
(20) (2) - Asparagine.....	+++	++		++		++

± = very faint growth.

* Methionine, serine, and proline not included in -B₁ medium.

TABLE 4
Growth of various strains of *B. alvei* and of *B. para-alvei* in media of different composition

ADDENDA TO BASAL MEDIUM	B. ALVEI STRAINS										B-PARA ALVEI	
	127	179	343	408	NS1	NS2	NS3	NS4	NS5	NS6		316
(1) 18 Amino acids.....	-	-	-	-	-	-	-	-	-	-	-	++
(2) 18 Amino acids + thiamine.....	+++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++
THIAMINE ADDED TO BASAL MEDIUM												
(3) Glycine + cystine + leucine*.....	+	+	++	+	+	+	++	-	+	++	++	++
(4) (3) + (NH ₄) ₂ SO ₄	+++	++	++	++	++	++	+++	+++	+	++	++	++
(5) 14 Amino acids†.....	+++	++	++++	++++	++++	+++	++++	+++	++	++++	++++	+++
(6) Acids in (1) - glycine.....	-	+	+	++	+	+	-	-	+	++	++	++
(7) Acids in (1) - cystine.....	-	++++	-	-	++++	-	++++	++	-	-	++++	+++
(8) Acids in (1) - leucine.....	+++	+	++++	++++	++	+++	+++	++++	+++	++++	++++	+++
(9) Yeast extract.....	+++	+++	++++	++++	++	+++	++++	++	++	++++	++++	+++

* Double the concentration given in text.

† See table 2 for the list of these acids.

The original *B. alvei* and *B. para-alvei* strains were also included in this experiment. All the strains of *B. alvei* required thiamine and could grow in a synthetic medium containing this vitamin and 18 amino acids. Again *B. para-alvei* grew in the absence of added thiamine but was stimulated by it. Strains of *B. alvei* developed poorly in a medium containing glycine, cystine, and leucine, but were apparently stimulated by the addition of $(\text{NH}_4)_2\text{SO}_4$ to these acids, indicating that inorganic nitrogen can replace certain amino acids if the more important ones are available. However, 14 amino acids permitted growth of most of the strains, which growth was distinctly superior to that produced in the $(\text{NH}_4)_2\text{SO}_4$ medium and practically equivalent to that obtained with 18 acids. Again, glycine was stimulatory for, or required by, all the strains of *B. alvei*; cystine for most; and leucine for several. In table 2 it was shown that leucine was stimulatory for strains 127, 179, 343, and 408, whereas in the last experiment it was found to stimulate only one of these (179). It should be pointed out, however, that in the first experiment leucine was omitted from a mixture of 14 acids, whereas in the experiment reported in table 4 it was omitted from a mixture of 18. It is quite possible that among the four omitted was one which could substitute for leucine in the medium containing 18 acids, thereby obscuring the effect of omitting it which appeared when only 14 acids were used. The best growth of *B. para-alvei* was obtained with 14 or 18 amino acids in the presence of thiamine, the organism developing moderately well in a medium containing the vitamin and glycine, cystine, and leucine. It was not stimulated appreciably by $(\text{NH}_4)_2\text{SO}_4$, nor was it affected by omission of cystine or leucine; however, when glycine was left out, growth was depressed.

DISCUSSION

As the following tabulation indicates, the most striking difference between the nutritional requirements of *B. alvei* and *B. para-alvei* strains is the essentiality of thiamine for the former as compared to its purely stimulatory effect on the latter in the amino acid medium.

ADDENDA TO BASAL MEDIUM	GROWTH OF	
	<i>B. alvei</i>	<i>B. para-alvei</i>
Casein hydrolyzate.....	0	0
Casein hydrolyzate + thiamine.....	+	+
Amino acids.....	0	+
Amino acids + thiamine.....	+	+

The growth of *B. para-alvei* in the amino acid medium devoid of thiamine may be due to contamination with this vitamin of one or more of the amino acids used, but in that case it might perhaps be expected that *B. alvei* would also grow in this medium, which is not the case. It is conceivable, too, that *B. para-alvei* is more sensitive to smaller amounts of thiamine such as might be found in contaminating traces than is *B. alvei*. The possibility that the former can synthesize the vitamin from the amino acids used, whereas *B. alvei* cannot, also suggests itself. This problem is being studied further. If it can be proved that *B. para-*

alvei can actually satisfy its thiamine requirements by synthesis instead of depending on an external supply as does *B. alvei*, then a fundamental physiological difference between these two organisms will have been brought to light, which, when combined with the morphological differences noted by Tarr (1936), may strengthen the case for separating these bacteria into two species.

ACKNOWLEDGMENT

The authors are indebted to Miss C. M. Clark for technical assistance.

SUMMARY

Bacillus alvei and *Bacillus para-alvei* required thiamine when grown in a casein hydrolyzate medium. In a synthetic medium containing amino acids, all strains of *B. alvei* tested required thiamine for growth, whereas strains of *B. para-alvei* produced moderate growth in the absence of this factor.

B. alvei grew in a simplified medium containing thiamine and 14 amino acids, of which glycine, leucine, and cystine were essential or stimulatory, depending on the strain; whereas *B. para-alvei* was stimulated by glycine. However, when thiamine was omitted from this amino acid medium, certain other acids such as valine, phenylalanine, isoleucine, and particularly cystine also became stimulatory or essential for the growth of the latter organism.

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