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Development of an Improved Chemically Defined Minimal Medium for *Listeria monocytogenes*

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A chemically defined minimal medium for *Listeria monocytogenes* has been developed by modification of Welshimer's medium. The growth factors required by *L. monocytogenes* Scott A are leucine, isoleucine, arginine, methionine, valine, cysteine (each at 100 mg/liter), riboflavin and biotin (each at 0.5 μg/ml), thiamine (1.0 μg/ml), and thioctic acid (0.005 μg/ml). Growth was stimulated by 20 μg of Fe^{3+} per ml as ferric citrate. Glucose (1%) and glutamine (600 mg/liter) are required as primary sources of carbon and nitrogen. Glucose could not be replaced by various organic acids or amino acids. Of several sugars tested, fructose, mannose, cellobiose, trehalose, maltose (weak), glycerol (weak), and the amino sugars glucosamine, *N*-acetylglucosamine, and *N*-acetylmuramic acid supported growth in the absence of glucose. Evidence was found that chitin and cell walls of starter bacteria (*Lactococcus lactis*) supported survival of *L. monocytogenes*, which suggests that the pathogen may obtain carbon and energy sources during colonization of some foods, such as cheeses, by assimilating bacteria or molds that are present.

Listeria monocytogenes is widespread in the environment and continues to present a potential health risk in raw, minimally processed, and some fermented foods. *L. monocytogenes* in cole slaw, milk, cheese, and possibly other foods, including undercooked wieners, sausages, poultry, and shellfish, has caused food-borne disease outbreaks (2, 3, 6, 7, 10). Since little is known of the capacity of *L. monocytogenes* strains to cause disease, the presence of the organism in ready-to-eat foods is considered unacceptable, and a zero tolerance of the organism in these foods is presently enforced by the Food and Drug Administration and U.S. Department of Agriculture. Contamination of foods with *L. monocytogenes* has been responsible for several class I recalls (9), which are defined as foods considered by the Food and Drug Administration or U.S. Department of Agriculture to pose a serious health hazard.

The occurrence of *L. monocytogenes* in low numbers in some raw and minimally processed foods may presently be unavoidable because of the pathogen's wide occurrence, resistance properties, and ability to colonize foods that vary in nutrient content. *L. monocytogenes* can utilize only a limited number of carbon sources for energy (8, 11), glucose being the preferred source. Milk, cheese, and other foods of animal origin contain small amounts of glucose. Therefore, *L. monocytogenes* must use alternate energy sources for growth in these products, possibly by degradation of polymeric substrates such as carbohydrates, proteins, and lipids. Little information on the growth and survival of this pathogen under nutritionally limited conditions is available.

To understand the ability of *L. monocytogenes* to grow and survive in widely diverse environments, it is desirable to determine the nutritional requirements in a chemically defined minimal medium. Several chemically defined media previously developed (5, 12, 13, 15, 16) were tested in our laboratory but did not support growth of *L. monocytogenes* Scott A and other strains (California [CA], Ohio [OH], V7, and ATCC 19115) through sequential subcultures. Other investigators have also reported poor growth of *L. monocytogenes* in chemically defined media (4). A minimal medium that supports good growth of *L. monocytogenes* is described in the present study.

The strain used for development of the medium was *L. monocytogenes* Scott A (serotype 4b, clinical isolate). Other strains were also used: V7 (serotype 1, milk isolate), CA (serotype 4b, isolated from Mexican-style cheese in the 1985 listeriosis outbreak in California), OH (isolated from recalled Liederkranz cheese manufactured in Ohio), ATCC 19115 (serotype 4b), and 28 strains isolated from dairy plants. All cultures were maintained at 4°C on brain heart infusion (BHI) agar slants and were transferred monthly. Strains were also kept at −70°C in BHI plus 20% glycerol. All glassware used for preparing media and culturing were acid washed and then rinsed with double-deionized (DD) water. To determine nutrient requirements, an initial inoculum was prepared by transferring a loopful of cells from BHI agar slants to BHI broth which was then incubated at 37°C for 18 h. Cells were harvested by centrifuging at 12,100 × *g* for 10 min, and pellets were washed twice in basal medium (Welshimer's broth [16]) and resuspended in 10 ml of the same medium. This suspension was used to inoculate (1% [vol/vol]) basal medium containing various combinations of growth factors. Cells were grown statically at 37°C in tubes containing 10 ml of medium. Growth was measured as the A_{660} in a Bausch & Lomb Spectronic 20 spectrophotometer; 1 mg (dry weight) of cells per ml corresponds to ca. 1.4 optical density units. Inocula for sequential transfers consisted of 1% (vol/vol) exponentially growing (20-h) cells.

Early in our studies of nutritional requirements, we noted that *L. monocytogenes* Scott A and other strains grew poorly in Welshimer's medium, reaching an A_{660} of 0.1 to 0.2 on a second transfer, and that growth was not sustained on consecutive transfers. Other investigators have reported similar findings (4). Limited growth was caused partially by limited buffering capacity, since the pH after incubation went to <5 at the buffer concentration (0.08 M phosphate) in the basal medium. Increasing the concentration of phosphate buffer from 0.08 to 0.2 M decreased the drop in pH to 6.3,

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TABLE 1. Amino acid requirements of *L. monocytogenes* Scott A

Concn (μg/ml)	Growth (A_{660})[a] in the presence of the following amino acid:								
	Leu	Ile	Val	His	Met	Arg	Trp	Cys	Gln
0	0.51	0.15	0.12	1.20	0.11	0.41	1.21	0.09	0.06
10	0.89	0.83	0.90	1.20	1.19	1.03	1.20	0.20	0.81
100	1.12	1.19	1.22	1.16	1.22	1.21	1.20	1.22	1.03
500	1.12	1.20	1.21	0.78	1.02	1.17	1.21	0.48	1.20
600									1.21
1,000									1.21

[a] Cells were grown for 30 h at 37°C. Complex medium supported growth to an absorbance of ~1.2.

but this was not sufficient for serial subculture. Growth in Welshimer's broth was reported to be stimulated by iron (14, 15). We found that growth in Welshimer's broth was stimulated by iron compounds, including hemin (50 μg/ml), hematin, ferric nitrate, and ferric citrate. Other iron compounds, including protoporphyrin IX, hematoporphyrin, and ferric chloride, did not promote growth. Of the various iron sources tested, ferric citrate promoted the best growth, and the optimum concentration was 20 μg/ml.

We next examined the amino acid and vitamin requirements of the Scott A strain. Welshimer (16) reported that *L. monocytogenes* required leucine, isoleucine, valine, methionine, arginine, cysteine, glutamine, histidine, and tryptophan. Using the Scott A strain, we confirmed the necessity of seven amino acids but found that histidine and tryptophan were not required (Table 1). Glutamine stimulated growth at levels higher than that needed for biosynthesis. Glutamine could not be replaced by ammonium ion. We confirmed that riboflavin, thiamine, biotin, and thioctic acid were required as growth factors, but optimal growth needed levels slightly higher than those previously reported by Welshimer (16) (Table 2).

The newly developed chemically defined medium (modified Welshimer's broth [MWB]) is described in Table 3. MWB supported growth of several known isolates and also of 28 isolates of *L. monocytogenes* obtained from the dairy environment. All strains grew in the medium at 5 and at 37°C. MWB also supported growth of *Listeria innocua* and *Listeria ivanovii*, which reached absorbances of 1.10 and 1.15, respectively.

L. monocytogenes is able to use a limited number of carbon sources. Pine et al. recently reported (8) that the availability of glucose determined the extent of growth in milk. They also reported that glucosamine or *N*-acetylglucosamine could be substituted for glucose in a semidefined medium and that lactose was used when the amino sugars were present. We found that growth of Scott A was strictly dependent on glucose and that concentrations higher than 0.5% were required for growth to exceed an A_{660} of >1. Various sugars, including fructose, mannose, trehalose, cellobiose, maltose (weak), and glycerol (weak), could substitute for glucose (Table 4), but several other sugars, including lactose, melibiose, sucrose, raffinose, sorbose, sorbitol, mannitol, galactose, galactose-1-phosphate, galactose-6-phosphate, arabinose, ribose, and xylose, did not support growth. Organic acids, including acetate, pyruvate, lactate, citrate, succinate, α-ketoglutarate, malate, and fumarate, also did not support growth in the absence of glucose. Interestingly, the amino sugars glucosamine, *N*-acetylglucosamine, and *N*-acetylmuramic acid supported growth of *L. monocytogenes*.

Promotion of growth by *N*-acetylglucosamine and *N*-acetylmuramic acid, which are components of both bacterial and fungal cell walls, suggested that digestion of bacteria or fungi present in foods may promote colonization and survival of *L. monocytogenes* in these foods. This idea was supported by the ability of *L. monocytogenes* to use trehalose and cellobiose, which are commonly found in fungi. We examined the influence of glucose, casein, chitin,

TABLE 2. Vitamin requirements of *L. monocytogenes* Scott A

Concn (μg/ml)	Growth (A_{660})[a] in the presence of the following vitamin:			
	Riboflavin	Thiamine	Biotin	Thioctic acid
0	0.13	0.36	0.17	0.30
0.1	1.16	1.04	0.82	1.17
0.5	1.17	1.17	1.16	1.17
1.0	1.15	1.18	1.01	1.17
2.0	1.07	1.06	0.96	0.98

[a] Growth represents the average of two trials. To minimize carryover, inocula for determining individual requirements were grown in medium lacking the vitamin. Pyridoxal HCl, choline chloride, *myo*-inositol, niacinamide, D-pantothenate (calcium), folic acid, and *para*-aminobenzoic acid were prepared as described by Siddiqui and Khan (12).

TABLE 3. Composition of Welshimer's broth and of MWB (minimal defined) media[a]

Component[b]	Amt/liter in Welshimer's broth	Amt/liter in MWB
KH_2PO_4	3.28 g	6.56 g
$Na_2HPO_4 \cdot 7H_2O$	15.48 g	30.96 g
$MgSO_4 \cdot 7H_2O$	0.41 g	0.41 g
Ferric citrate	0 g	0.088 g
Glucose	10.0 g	10.0 g
L-Leucine	0.1 g	0.1 g
L-Isoleucine	0.1 g	0.1 g
L-Valine	0.1 g	0.1 g
L-Methionine	0.1 g	0.1 g
L-Arginine	0.1 g	0.1 g
L-Cysteine	0.1 g	0.1 g
L-Histidine	0.1 g	0 g
L-Tryptophan	0.1 g	0 g
L-Glutamine	0.6 g	0.6 g
Riboflavin	1.0 mg	0.5 mg
Thiamine	1.0 mg	1.0 mg
Biotin	0.1 mg	0.5 mg
Thioctic acid	0.001 mg	0.005 mg

[a] MWB was prepared as follows to prevent precipitation or denaturation of essential nutrients. The buffer component salt A ($Na_2HPO_4 \cdot 7H_2O$ [154.8 g] and KH_2PO_4 [32.8 g]) was dissolved in DD water, and the volume was brought up to 500 ml; salt B, $MgSO_4 \cdot 7H_2O$ (4.09 g), was dissolved in DD water, and the volume was brought up to 100 ml. Both salt A and salt B solutions were autoclaved for 15 min. Glucose was prepared as a 20% stock solution and filter sterilized. Amino acids required for biosynthesis (leucine, isoleucine, arginine, methionine, and valine) were mixed in 100-fold excess (10 g/liter), steamed to dissolve in DD water, and filter sterilized after cooling. Cysteine (5 g/liter) and glutamine (30 g/liter) were dissolved in 50-fold excess and filter sterilized. Cysteine and glutamine must be added fresh to the medium. A 100-fold stock solution of ferric citrate was prepared by mixing 0.439 g in hot DD water and bringing the volume up to 100 ml. Vitamins were also prepared as 100-fold solutions (per liter): riboflavin (50 mg) was dissolved in 1 N formic acid, biotin (50 mg) was dissolved in hot water and cooled to room temperature, thiamine (100 mg) was dissolved in DD water, and thioctic acid (0.5 mg) was dissolved in a small volume of 70% ethanol and diluted with DD water. The vitamin solutions were filter sterilized and kept refrigerated.

[b] Certain components were stimulatory but not required. Nucleotide precursors (cytosine, guanine, adenine, and thymine, each at 2.5 mg/liter) and hemin (50 mg/liter) were stimulatory.

TABLE 4. Carbon sources supporting growth of *L. monocytogenes* in minimal medium

Energy source[a]	Growth (A_{660}) after 24 h
Glucose	1.18
Fructose	1.09
Mannose	1.10
N-Acetylglucosamine	0.99
N-Acetylmuramic acid	0.88
Glucosamine	1.00
Cellobiose	1.10
Trehalose	0.96
Maltose	0.10[b]
Glycerol	0.11[b]

[a] No growth was obtained with various sugars (including lactose, melibiose, sucrose, raffinose, sorbose, sorbitol, mannitol, galactose, galactose-1-phosphate, galactose-6-phosphate, glucose-1-phosphate, glucose-6-phosphate, arabinose, ribose, and xylose, organic acids (including acetate, pyruvate, lactate, succinate, fumarate, α-ketoglutarate, malate, citrate, and isocitrate), chitin, and essential and nonessential amino acids as energy sources.
[b] Growth (A_{660}) after 48 h.

and bacterial cell walls on survival. Survival of *L. monocytogenes* in MWB or in 0.067 M phosphate buffer (pH 6.6) decreased markedly compared with that in MWB without a carbon source (Fig. 1). Chitin or cell walls prepared from *Lactococcus lactis* also supported survival (Fig. 1). These data support the interesting possibility that *L. monocytogenes* can digest the cell walls of the microflora of bacteria and obtain carbon and energy during the colonization of foods. Although chitin and cell walls promoted survival, we have not been able to obtain growth on these carbon sources in MWB. Further work is needed to determine the role of cell wall digestion during colonization and survival in foods.

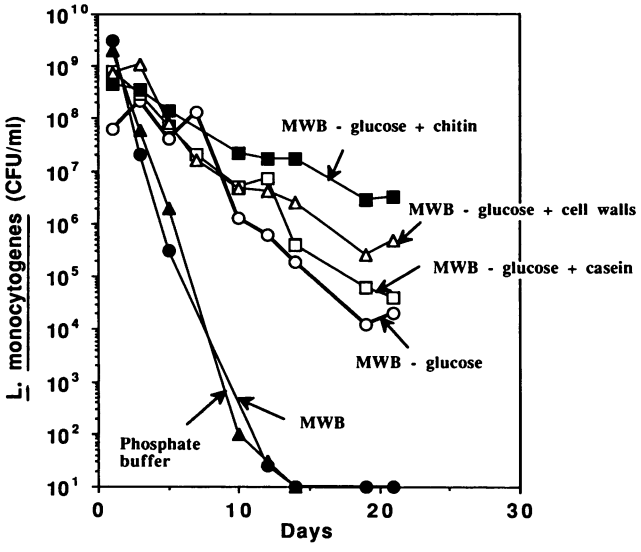

FIG. 1. Survival of *L. monocytogenes* Scott A incubated in MWB and in MWB minus glucose plus various carbon and energy sources. Cell walls from *Lactococcus lactis* were prepared by the method of Berenguer et al. (1).

L. monocytogenes is relatively fastidious in nutrient requirements, and it requires a sugar, seven amino acids, four vitamins, and a suitable source of iron for growth. The medium described in this study should be useful for determining the nutrients involved in food colonization and the survival and resistance properties of the pathogen.

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